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THE EFFECTS OF WATER-SOLUBLE CARBON MONOXIDE-RELEASING MOLECULES (CO-RMs) ON VASCULAR TONE

by

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DEDICATION

To my parents

My wife

My brothers and sisters

And to my large family

I dedicate this work.

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Summary

Carbon monoxide (CO) is an important signalling messenger in mammalian cells as it participates in a variety of physiological processes including vessel tone regulation. Dr. Motterlini's group has discovered a new class of molecules which have the ability to carry and deliver CO to physiological systems. These molecules were termed CO-releasing molecules (CO-RMs) and they are an excellent tool to investigate the biological role of CO in the vasculature and other systems.

The major focus of the research presented in this report was to evaluate the effects of different water soluble CO-RMs on vascular tone. For this purpose, an aortic ring preparation model was used to assess the vasodilatory properties of CORM-3 and CORM-A1, the first two water-soluble CO-RMs to be identified, and the cellular targets involved in this effect. CORM-3 is a transition metal carbonyl that liberates CO very rapidly ($t_{½}$=1-5 min) in physiological solutions, whereas CORM-A1 is a boron-containing carbonate with a much slower rate of CO release ($t_{½}$=21 min at pH=7.4). In the current studies CORM-3 induced a rapid endothelium-dependent vasorelaxation, whereas CORM-A1 elicited gradual endothelium-independent vasorelaxation. The inactive form of both CO-RMs, in which CO has been deliberately depleted, did not exert vasorelaxation indicating a direct involvement of CO liberated from the compounds in the observed vasorelaxation. The vasorelaxation induced by both molecules was enhanced and attenuated by an activator and inhibitor of guanylate cyclase (sGC), respectively. CORM-3-mediated vasorelaxation was completely abolished by non-selective inhibitors of potassium channels (K^+), and partially attenuated by inhibition of ATP dependant (K_{ATP}) potassium channels. In contrast, CORM-A1-mediated

vasorelaxation was partially attenuated by non selective inhibition of K^+ and by inhibition of voltage dependent (K_V) potassium channels. Even at concentrations higher than that used to induce significant vasorelaxation, both CO-RMs had no noticeable effect on the viability of rat aortic smooth muscle cells (A7r5) *in vitro*. CORM-319, a new water soluble iron containing CO-RM, also induced significant vasorelaxation and was relatively safe to cultured SMCs compared to other non-water soluble iron containing CO-RMs that were extremely toxic. In summary, our data reveal that the CO-RMs examined in this project are promising CO carriers that could be further modified for optimal therapeutic applications. In addition, our data demonstrate the significant effect imposed by the chemical structure and kinetics of CO release on the pharmacological activity of various CO-RMs.

List of abbreviations

4-AP:	4-aminopyridine
APA:	apamine
ATP:	adenosine triphosphate
BK_{Ca}:	big conductance calcium activated K^+ channels
cAMP:	cyclic adenosine monophosphate
cGMP:	cyclic guanosine monophosphate
($[Ca^{+2}]_i$):	intracellular Ca^{2+} concentration
CHB:	charybdotoxin
CO:	carbon monoxide
CO-RMs:	carbon monoxide releasing molecules
DMSO:	dimethyl sulfoxide
E^+:	endothelium intact
E^-:	endothelium denuded
E-CO:	exhaled CO
EDHF:	endothelium dependant relaxing factor
GC:	guanylate cyclase
GLI:	glibenclamide
GTP:	guanosine triphosphate
HO:	haem oxygenase enzyme
HO-1:	haem oxygenase-1
HO-2:	haem oxygenase-2
K^+:	potassium channels
K_{ATP}:	ATP dependant K^+ channels
K_{Ca}:	calcium activated K^+ channels

KCl:	potassium chloride
K_{ir}:	inward rectifier K^+ channels
K_V:	voltage sensitive K^+ channels
L-NAME:	N^G-nitro-L-arginine-methyl ester
NANC:	neurally evoked nonadrenergic noncholenergic relaxation
NO:	nitric oxide
NOS:	nitric oxide synthase
ODQ:	1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-One
ORD:	oxygen radical disease
pGC:	particulate guanylate cyclase
PDE:	phosphodiestarase
PDGF:	platelet derived growth factor
Phe:	phenylephrine
PKA:	cAMP-dependant protein kinase (PKA)
PKG:	cGMP-dependant protein kinase
sGC:	soluble guanylate cyclase
SK_{Ca}:	small conductance Ca^{2+} activated K^+ channels
SMCs:	smooth muscle cells
SnPP:	protoporphyrin-IX
TEA:	tetraethylamonium
YC-1:	benzyl indazole derivative

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1 Introduction

1.1 Historical background on carbon monoxide: old enemy or new friend?

Carbon monoxide (CO), a gas with a bad reputation, has been known for a very long time. Aristotle is attributed with the first recognition of CO toxicity in the third century BC when he described it as "Coal fumes lead to heavy head and death" (Morse and Sethi, 2002). In the 19th century a French physician, Leblanc, established Aristotle's observation by identifying CO as the toxic molecule in coal gas (Morse and Sethi, 2002). The formation of CO in the body was demonstrated in 1952 by Sjostrand, who reported that decomposition of haemoglobin *in vivo* led to CO production (Sjostrand 1952; Marks *et al.,* 2002 a). In 1968 haem oxygenase (HO), the enzymatic source of CO was identified (Tenhunen *et al.,* 1968), and later on two isoforms of the haem oxygenase enzyme (HO-1 and HO-2) were purified and identified in rat liver (Maines *et al.,* 1986). Haem oxygenase-1 (HO-1), the inducible form, and haem oxygenase-2 (HO-2), the constitutive form, cleave and oxidize the α-methene bridge of the haem molecule giving rise to equimolar amounts of biliverdin, CO and iron (Maines *et al* .,1997).

CO is a tasteless, colourless, odourless gas; some physical characteristics of this gas are shown in Table 1.1 (Piantadosi *et al.,* 2002; Ryter and Otterbein, 2004). CO is usually generated by the incomplete combustion of organic matter such as propane, coal, natural gas, wood and gasoline (Ryter and Otterbein, 2004). The average global levels of CO in the atmosphere are estimated to be 0.19 parts per million (ppm). 90% of this gas comes from natural sources including ocean microorganism generation and the remaining part is produced by human activity (Motterlini *et al.,* 2002b).

Physical properties of CO

Molecular Weight	28.01
Boiling Point	-191.5 ^0C
Melting Point	-205 ^0C
Solubility (H2O)	3.3 ml 100 ml^{-1} @ 0 ^0C
	2.3 ml 100 ml^{-1} @ 20 ^0C
Density (vapour)	0.968 air = 1
Specific Gravity	1.250 gL^{-1} @ 0 ^0C
Conversion Factor	1 ppm = 1.25 mg m^{-3} at 25 ^0C

Table 1.1: Physical properties of CO (adapted from Ryter and Otterbein. 2004).

Carbon monoxide has been considered as the most common lethal poison in every community yet studied, and accounts for more morbidities than all other non-prescribed poisons combined (Raub *et al.* 2000.; Gorman *et al.,* 2003). The toxicity of CO is attributed to its high affinity for haemoglobin as the affinity of haemoglobin to CO is 240 times that for O_2 (Morse and Sethi, 2002; Omaye, 2002). In addition, the CO haemoglobin ligand on any one of the four oxygen binding sites of the haemoglobin molecule prevents oxygen at other sites of haemoglobin from being easily released to peripheral body tissues (Morse and Sethi, 2002; Omaye 2002). As a consequence the haemoglobin dissociation curve is shifted to the left and tissue hypoxia is further exacerbated. CO also binds to and inhibits the activities of other haemoproteins such as cytochrome c oxidase, cytochrome P450 and myoglobin, although this inhibitory effect would be likely to occur at very high levels of CO exposure (Morse and Sethi 2002; Ryter and

Otterbein, 2004).

Interestingly, over the last few years evidence has accumulated showing that HO enzymes and their by-products, particularly CO, are important factors in cellular metabolism. Thirteen years ago after tracking the story of CO and comparing it to nitric oxide (NO) gas, Barinaga predicted that CO would provide a research interest to fully occupy laboraties in future (Barinaga *et al.,* 1993). If the interest of the scientific community can be estimated by the number of publications dealing with a particular subject, then the prediction of Barinaga surely turned out to be true (Morse and Choi 2002) **(Figure 1.1).**

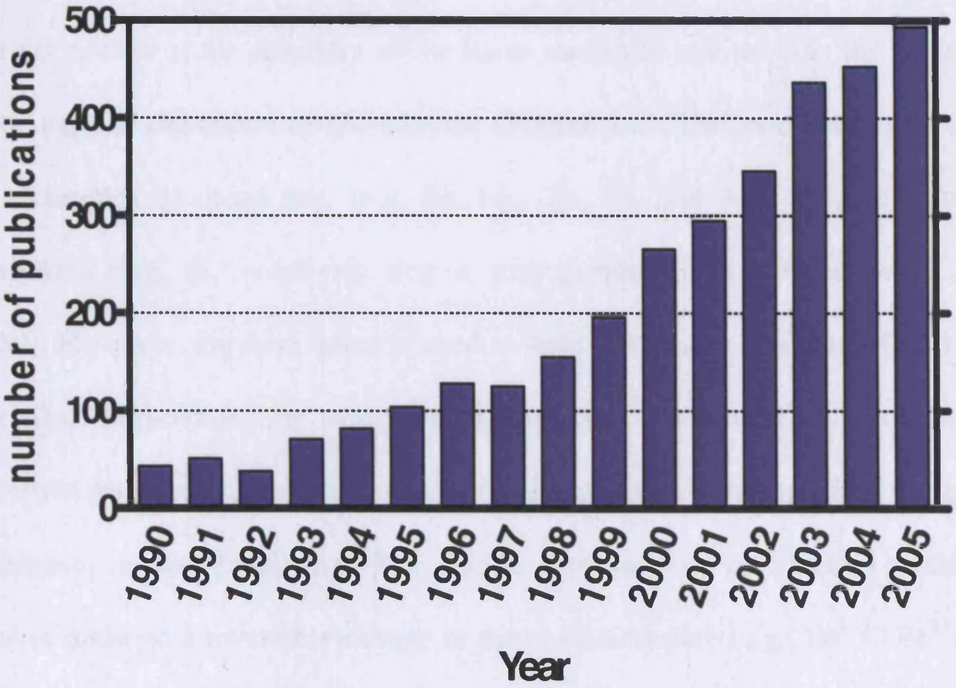


Figure 1.1. Number of publications concerning HO-1 catalogued by PubMed from 1990 to 2005 (adapted from Morse and Choi 2002).

1.2 The Haem Molecule

The haem molecule plays many crucial biological functions (Wagener *et al.,* 2003). It interacts with various inactive apo-haem proteins yeilding functional haem proteins such as haemoglobin, myoglobin, cytochromes, catalases and peroxidases enzymes. Additionally, haem itself may influence the expression of many genes and regulates differentiation and proliferation of various cell types (Wagener *et al.*, 2003). On the other hand, excess of free haem molecule can cause cell damage and tissue injury since haem catalyzes the formation of reactive oxygen species (ROS), which cause oxidative stress. Therefore, the HO system is a crucial defence mechanism against free haem-mediated oxidative stress (Wagener *et al.,* 2003).

A brief review of the structure of the haem molecule will provide the basis to understand the nature of HO enzyme (**Figure 1.2**). The term haem refers to a complex of metal ions (e.g, Fe, Mg, Zn, Cr, and Ag) chelated in a porphyrin ring; the porphyrin ring is protoporphyrin IX (Maines *et al.,* 1988). However, the term haem is used to refer to the iron complex of such chelates. Porphyrins, in which a metal ion is chelated, are cyclic tetrapyrroles in which the four pyrrole rings are attached through four meso (methane) bridges labelled α, β, γ, and δ. The capacity of chelated metal ions to undergo a reversible change in the oxidation state (e.g., $Fe^{2+} \leftrightarrows Fe^{3+}$) makes haem compounds effective biological catalysts (Maines *et al.,*1988). Several types of haem which differ in the composition of the side chains of the pyrrole rings have been identified in nature. Haem type *b* identified in higher animals is the most ubiquitous and comprises the prosthetic moiety of all haem-proteins except cytochromes type *a* and *c*. Haem a and c form the prosthetic moiety of cytochromes *a* and *c* respectively (Maines *et al.*,1988).

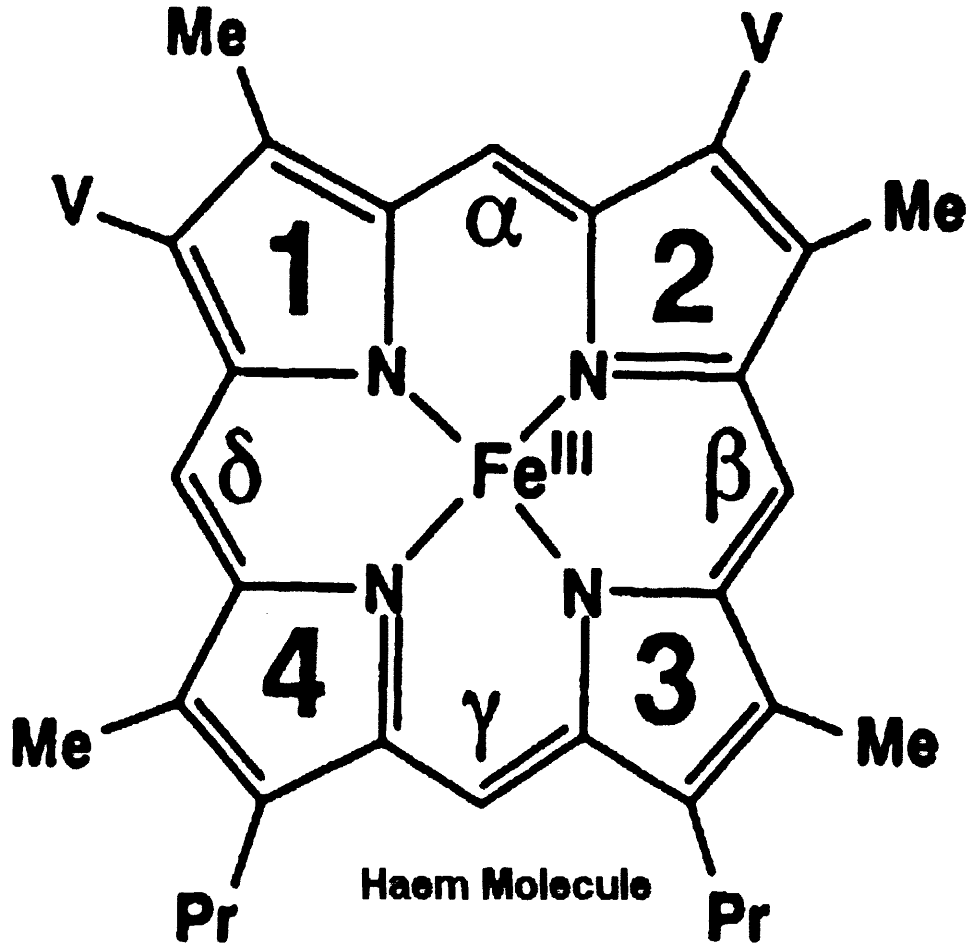


Figure 1.2 Structure of protoporphyrin IX (Haem b). (adapted from Maines *et al.*, 1997)

1.3 The Haem Oxygenase System

HO is the rate limiting enzyme in the catabolism of haem (Maines *et al.*, 1988). It breaks down the porphyrin ring to yield equimolar amounts of biliverdin, free iron (Fe^{2+}) and CO (**Figure 1.3**). Biliverdin is then rapidly converted into bilirubin by biliverdin reductase (Maines *et al.*, 1988). The HO system requires the activity of microsomal NADPH-cytochrome P450 reductase, which transfers an electron from NADPH to haem, and utilizes molecular oxygen for cleavage of haem. HO specifically cleaves the α-methene bridge of the haem b molecule (Maines *et al.*, 1988; Maines *et al.*, 1992).

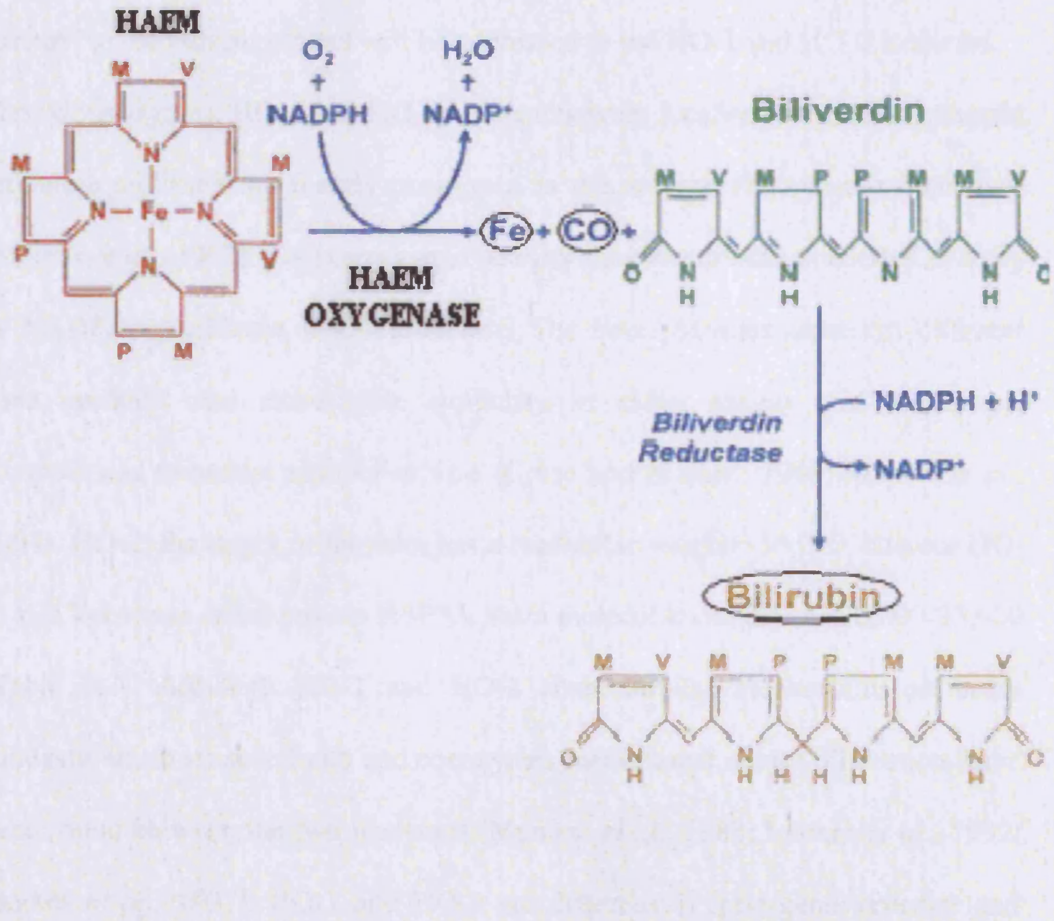


Figure 1.3. Products of haem degradation by haem oxygenase enzyme (adapted from Durante *et al.*, 2003).

In mammals three isoforms of HO proteins have been identified. The first isoform, now known as HO-1, was first described in 1969 (Tenhunen *et al.*, 1968). Later on, the second isoform, now known as HO-2, was purified and identified (Maines *et al.*, 1986; Trakshel *et al.*, 1986). The third isoform (HO-3), was identified around eight years ago (McCoubrey *et al.*, 1997). HO-3 has been found only in rats and it has been proposed that it could have originated from retrotransposition of the HO-2 gene (Scapagnini *et a.,*. 2002). In contrast to other two HO isoenzymes, HO-3 is a poor haem catalyst and it is unlikely to work as haem degrading enzyme but it might be involved in haem binding (McCoubrey *et al.*, 1997). Because HO-3 has no reported biological activities and it is not expressed in humans (Abraham and Kappas. 2005), the general referral to "HO system" in the current project will be addressed to the HO-1 and HO-2 isoforms.

The two isozymes, HO-1 and HO-2, are exclusively localised in the endoplasmic reticulum and they are mainly positioned in the smooth endoplasmic reticulum (Maines *et al.*, 1992). To express their activity they require the concerted activity of NADPH-cytochrome P-450 reductase. The two isozymes represent different gene products and share little similarity in either amino acid sequences, composition, transcript number or size (Cruse and Maines, 1988; Maines *et al.*, 1997). HO-2, the larger of the two, has a molecular weight ~36,000 whereas HO-1, also known as stress protein HSP32, has a molecular weight of ~30,000-33,000 (Table 1.2). Although HO-1 and HO-2 share similar mechanisms as haem catalysts, substrate specificity and coenzymes requirement, major differences have been found between the two isozymes (Maines *et al.*, 1988; Maines *et al.*, 1992; Maines *et al.*, 1997). HO-1 and HO-2 are different in their gene structure and organisation, in chromosomal localisation, regulatory mechanisms and tissue distribution. Both HO-2 and HO-1 are single-copy genes but HO-2 is encoded by two transcripts (~1.3 and ~ 1.9 kb) whereas the HO-1 protein is the transcription product of a single message of ~1.8 kb (Maines *et al.,* 1997). A 24-amino acid-long segment of HO-1 and HO-2 is conserved among all haem oxygenases. The 24-amino acid segment forms a hydrophobic pocket (haem pocket) that binds haem pyrrole rings. A part of this segment is now recognised as the HO signature (Maines *et al.,* 1997).

1.3.1 Regulation and distribution of haem oxygenase nzymes

In fact HO is expressed in almost all life forms from prokaryotic bacteria and fungi to plants and human (Wagener *et al.,* 2003). When the HO-1 and HO-2 isozymes are evaluated by assessing their protein and message levels they show different patterns of distribution and development (Maines *et al.,* 1997). Most cells, under normal physiological conditions, express low or undetectable levels of HO-1 protein, while HO-2 protein is constitutively expressed (Wagener *et al.,* 2003). The only exception is spleen in which under normal, unstressed conditions, HO-1 is the predominant form (Maines *et al.,* 1997). Although it is not constitutively expressed in most tissues under normal physiological conditions, HO-1 transcription is up-regulated by a huge number of stimuli such as oxidative stress, growth factors, disease states, hormones, dietary antioxidants and some physiological changes (Table 1.3) (Maines *et al.,* 1997; Kim *et al.,* 2005). There is no harmony among HO-1 inducers, which have diverse chemical structures varying from proteins to small organic compounds to a gas molecule (NO). Actually, there is no enzyme identified to date that is induced by so numerous stimuli of diverse nature as is HO-1 (Maines *et al.,* 1997). Unlike HO-1, the inducers of HO-2 are very limited and include adrenal glucocorticoids, developemental factors, opiates and possibly nitric oxide (NO) (Maines and Panahian, 2001). The activity of HO-1 is high even at low concentrations of haem and it increases with rise of haem concentrations within physiological limits (Wagener *et al.*, 2003). On the other hand, HO-2 has a low level of activity at low haem concentrations and even its maximum activity is limited to less than 10 % that of HO-1. Specific activity of HO, which reflects the activity of both HO-1 and HO-2, varies greatly in different organs; the highest HO activity has been reported in the spleen, brain and testes (Maines *et al.*,1988; Maines *et al.*,1997).

TABLE 1.2; Summary of various functions of HO isoforms, their distribution and gene regulation (adapted from Wagener *et al.* 2003; Yachie *et al.*, 2002).

	HO-1 (hsp32) Isoform	HO-2 Isoform	HO-3 Isoform
Molecular weight	~32 kD	~36kD	~33kD
Physiological role	Haem degradation Modulation of vascular tone and liver perfusion Neural signaling Anti-inflammatory action Down-modulation of adhesion molecule expression	Haem degradation Haem binding Maintenance of vascular tone Neural signaling	Haem binding
Constitutive tissue	Spleen, liver	Most tissues e.g., brain, retina, liver, spleen, testis, lungs, vascular	Most tissues
Inducers of expression	Very diverse, e.g., oxidative stress, cytokines, heavy metals, hypoxia, nitric oxide, heat shock, haem and UV	Adrenal corticoid, opiates	Unknown
Enzyme activity	$K_m = 0.24\ \mu M$ $V_{max} = 3.4\ \mu mol/mg/h$	$K_m = 0.67\ \mu M$ $V_{max} = 0.24\ \mu mol/mg/h$	

TABLE 1.3; Various stimuli induce HO-1 production (adapted from Kim *et al.*, 2005)

Disease state	**Oxidative stress**
Ischaemia reperfusion	Endoplasmic reticulum stress
Inflammation	Heavy metals
Immune dysfunction	Oxidized LDL
Transplantation	Hydroperoxide
Myocardial infraction	Dieldrin
Atherosclerosis	Cigarette smoke extract
Hypertension	Alcohol
Vascular restenosis	
Glomerulonephritis	
Asthma	**Dietary antioxidant/natural product**
Alzheimer's disease	Synthetic terpenoid
Hormone	Quercetin/flavonoid
Estrogen	Mycotoxin
Prolactin	Ferulic acid/ethyl ferulic acid
Adrenocorticotrophin	Zerumbone
Atrial natriuretic peptide	Selegiline
Angiotension	Turpentine oil
Progesterone	3-0-caffeoyl-methyl quinic acid
Glucocorticoid	Diallyl sulphide
Medicines/chemicals	Chalcone/rosolic acid
Immunosuppressant/anti-inflammatory	Taurine
IL-10	Curcumin
Aspirin/salicylic acid	Cafestol/kahweol
Rapamycin	Avicins
Antioxidant	Resveratrol
Vit E	**Physiological changes**
Miscellaneous	Hypertonic saline
Dopamine	Transient glucose deprivation
Nicotine	Acidosis
Cysteamine	Nitric oxide
UV irradiation	Cytokine
Co-protoporphyrin	Mechanical shear stress

1.4 Haem Oxygenase Metabolites

Over a long time, HO has been thought to be only involved in the breakdown of haem from senescent red blood cells or denatured haem-proteins and the degradation products free iron, biliverdin/bilirubin, and CO were considered "toxic waste" materials (Johnson *et al.,* 1999; Morse and Choi, 2002). However, the attitude toward HO metabolites has changed dramatically over the last two decades and hundreds of studies investigating the biological roles of these compounds, especially CO, have published every year. The biological roles of bilirubin and iron will be briefly described in the following two sections, and then a detailed study of the beneficial role of CO in biological systems will be presented.

1.4.1 Bilirubin

Bilirubin is formed from the catalysis of biliverdin by the cytosolic enzyme biliverdin reductase (Kutty and Maines 1981; Maines *et al.,* 1988). This enzyme is considred as one of the most unique proteins in the animal kingdom because it has two pH optima, 7.0 and 8.7 (Maines *et al.,* 1988). At pH 7.0, biliverdin reductase has clear preference for the NADH cofactor whereas at pH 8.7 it has preference for the NADPH cofactor. At pH 7.4, biliverdin reductase has low activity with either factor (Kutty and Maines 1981; Maines *et al.,* 1988). The daily generation of bilirubin in adult humans is around 300 mg and because it is sparingly soluble in water it is tightly bound to albumin in the circulation (Stocker *et al.,* 1990). The physiological concentration of plasma bilirubin is between 5 and 20 μM, practically all of which is unconjugated pigments bound to albumin. Circulating bilirubin is taken up by hepatocytes where it is transformed into water soluble conjugated bilirubin. The concentration of conjugated bilirubin in the liver ranges from 20 to 40 μM and becomes more concentrated (0.35 to 4 mM) after excretion into bile. Concentrated conjugated bilirubin is excreted into small intestine where it is transformed into urobilinogens which are then either reabsorbed or excreted in the faeces (Stocker *et al.*, 1990).

Bilirubin has been shown to have antioxidant effects (Stocker *et al.,* 1987; Stocker *et al.,* 1987; Neuzil and Stocker 1993). Bilirubin efficiently scavenges peroxyl radicals and when the experimental concentration of oxygen is < 2 %, the antioxidant activity of bilirubin is more effective than alpha-tocopherol, which has long been regarded as the best antioxidant of lipid peroxidation (Stocker *et al.,* 1987). In one clinical study, 25 preterm infants who have oxygen-radical disease (ORD) (e.g., bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage and retinopathy of prematurity) were shown to have lower bilirubin levels compared to 57 control infants who did not have disorders related to ORD (Hegyi *et al.,* 1994). Bilirubin and biliverdin were also reported to have cytoprotective antioxidant effects in hepatocytes (Wu *et al.,* 1991a).

In addition to its antioxidant effect, bilirubin plays a protective role against some cardiovascular disorders (Wu *et al.,* 1991b; Wu *et al.,* 1994; Hopkins *et al.,* 1996; Clark *et al.,* 2000). It was reported that bilirubin, whether endogenously produced from haem degradation or exogenously administrated, significantly ameliorated myocardial functions and minimized both infarct size and mitochondrial damage on perfusion of isolated hearts (Clark *et al.,* 2000). Furthermore, in another study, mildly elevated serum bilirubin was associated with decreased risk of early familial coronary artery disease (Hopkins *et al.,* 1996). In yet another larger study, where 1741 subjects were screened for carotid plaque, it was found that the serum bilirubin level was inversely correlated with the presence of carotid plaque

(Ishizaka *et al.,* 2001).

Although collective evidence from animal and human studies indicates that bilirubin is a significant physiological cytoprotectant, this protective effect of a moderate rise of bilirubin does not change the well-established dangers of brain damage (kernicterus) associated with a major rise of serum bilirubin (Sedlak and Snyder, 2004).

1.4.2 Iron

Unlike CO, very little attention has been devoted to ferrous iron the product of haem degradation (Snyder and Baranano, 2001). It has been revealed that cells must have protective mechanisms to deal with iron released by HO because ferrous iron is extremely toxic even at low concentration (Snyder and Baranano, 2001). It is fundamental to the Fenton reaction that produces hydroxyl free radicals, one of the most toxic biological species, which play a major role in cellular death in septic shock and strokes (Snyder and Baranano, 2001). However, the ferrous iron generated from haem degradation by HO also induces the synthesis of ferritin, an intracellular protein present in species ranging from bacteria to humans, which binds and stores free iron inside the cell and has well known cytoprotective properties (Eisenstein *et al.,* 1991; Balla *et al.,* 1992). In addition to promoting the synthesis of ferritin, production of ferrous iron also leads to rapid expression of an ATPase pump that may mediate the efflux of cellular iron for iron re-utilization, and therefore reduces iron accumulation within cells, thus protecting cells under stress (Ferris *et al.,* 1999). The role of ferritin in mediating the cytoprotection of HO-1 is still arguable as it sometimes appears to substitute for HO-1 cytoprotection and sometimes does not (Otterbein *et al.,* 2003). It was suggested that in endothelium and perhaps other cell types, HO-1 serves mainly to degrade haem to free iron which in turn induces the synthesis of ferritin, which is likely the proximate protective against oxidative damage (Balla *et al.,* 1992). On the other hand, it has been reported that haemoglobin-induced protection against the inflammatory consequences of lipopolysaccharide administration is dependent on HO-1 not ferritin induction (Otterbein *et al.,* 1997). The respective contributions of ATPase pumps, that pump iron from the cell, versus ferritin, which sequesters free iron within the cell, to the overall cytoprotective effects of HO are not clear but both mechanisms might be involved in a vital way to the overall HO antioxidant effect (Otterbein *et al.,* 2003).

1.5 Carbon monoxide (CO)

Sources and quantification of endogenous CO, beneficial roles of CO in different biological systems, particularly blood vessels, interaction with NO in vascular tissues, and the development of new CO releasing molecules (CO-RMs) will be discussed thoroughly in the following sections.

1.5.1 Sources of endogenous CO

Under normal physiological conditions there are two sources of endogenous CO production, which include either haem or non-haem sources (**Figure 1.4**) (Vreman *et al.,* 2002). The main sources of CO come from haem degradation catalysed by HO. The majority of this haem (~79%) comes from senescing red blood cells whereas the remaining haem is derived from the turn over of other haem-proteins such as myoglobin, cytochromes, catalase and others (Vreman *et al.,* 2002). Around 14% of CO produced in the body comes from non-haem processes, such as lipid peroxidation, photooxidation, xenobiotic activity in blood and others (Vreman *et al.,* 2002).

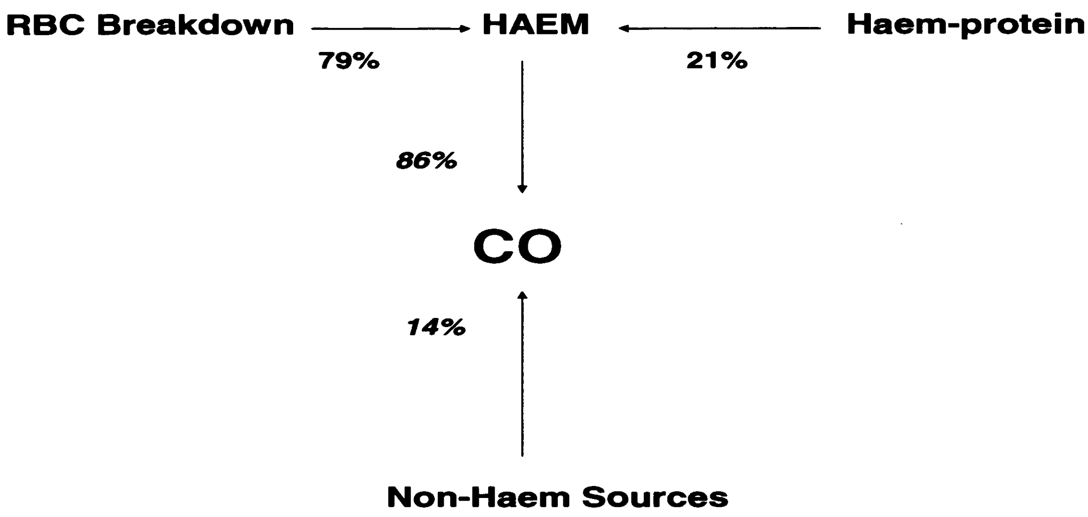


Figure 1.4. Sources of endogenous CO (adapted from Vreman *et al.*, 2002)

1.5.2 Measurement of CO formation in biological systems

CO generated endogenously has been measured in various biological systems using a diversity of analytical procedures which include laser sensor-infrared absorption, gas chromatography-mass spectroscopic detection, and gas chromatography-reduction gas detection, and formation of ^{14}CO from ^{14}C-haem formed following [2-^{14}C] glycine administration (Marks *et al.,* 2002). Although different methods have been used to measure CO generation by different tissues, the rate of CO formation varies over a short range from 0.28 nmol/mg of protein/h in rat liver perfusate and cultured rat olfactory receptors neurons down to 0.029 nmol/mg of protein in chronic villi of full term human placenta (Marks *et al.,* 2002). The high levels of CO production in liver and certain brain areas might reflect the high level of HO activity in these organs (Maines *et al.,* 1997; Marks *et al.,* 2002)

1.6 Biological Roles of CO

1.6.1 CO and the lung

Because lung is an organ that provides communication between the environment and the circulation it represents an ideal environment for studying oxidative stress (Morse and Choi, 2002). Oxidative stress results from imbalance between oxidants and antioxidants which happens either from a rise of oxidants and/or decrease in antioxidants (Rahman *et al.,* 1996). The release of reactive nitrogen species and oxygen species (RNS and ROS, respectively) from inflammatory leukocytes and also from airway epithelial cells is the main source of increased oxidant load in inflammatory lung diseases (Horvath *et al.,* 2001). Inhaled oxidants in cigarette smoke and air pollutants also contribute to oxidative stress. The lung is armed with a number of antioxidant systems which vary in their significance in various sites in the respiratory tract (Horvath *et al.,* 2001)

HO-1 is induced in the lung by many stresses and has been involved in the preservation of homeostasis against oxidative injury and there is evidence that CO released from haem degradation by HO-1 mediates at least some of the protective properties of HO-1 in the lung (Morse and Choi, 2002). It has been reported that animals exposed to low concentrations of CO showed a noticeable tolerance to lethal concentrations of hyperoxia *in vivo* (Otterbein *et al.,* 1999). In the same study exogenous CO totally protected against hyperoxia-induced lung injury in rats in whose endogenous HO enzyme activity was inhibited. This result indicates the role of CO in mediating HO-1 antioxidant effects in the lung. In another study, where the functional role of CO in asthma was assessed using an aeroallergen model, the rats exposed to CO for 48 h had lower levels of some proinflamatory factors than control rats (Chapman *et al.*, 2001). These data suggest a putative immunoregulatory role for CO in aeroallergen-induced inflammation in mice.

1.6.2 Exhaled CO (E-CO) marker

In humans exhaled CO (E-CO) is formed primarily from both systemic haem metabolism, which generates CO in different tissues, and localised (lung) haem metabolism which results from the combined action of HO-1 and HO-2 enzymatic activity (Slebos *et al.,* 2003). As CO, either endogenously produced or inspired, is eliminated exclusively by respiration, E-CO can be used as a marker to monitor the increase in exogenous sources of CO such as smoking or air pollution (Horvath *et al.,* 2001; Morse and Sethi 2002; Slebos *et al.,* 2003). It can be also used to monitor the increase in endogenous CO production resulting from systemic and local pulmonary inflammation (Horvath *et al.,* 2001; Morse and Sethi, 2002; Slebos *et al.,* 2003).

Indeed E-CO has been reported to increase in a number of systematic and pulmonary pathological conditions such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), infectious lung disease and diabetes (Antuni *et al.,* 2000; Horvath *et al.,* 2001; Morse and Sethi, 2002). During exacerbations of chronic disease, CO production, estimated by E-CO, may rise over what is already an elevated baseline (Antuni *et al.,* 2000; Morse and Sethi, 2002). For example, E-CO in patients with cystic fibrosis disease was higher than that in control, but it increases further during exacerbation of the disease, and goes back to baseline again after seven days of antibiotic treatment (Antuni *et al.,* 2000). Similar to the early era of measurement of exhaled NO, a standardization in techniques and agreement on background correction need to be achieved for E-CO measurements, to draw proper conclusions in this area of investigation (Slebos *et al.,* 2003).

1.6.3 CO and inflammation

Inflammation is a defence system used by organisms to protect them from pathogenic invaders, to remove damaged cells after injury and to prevent further damage (Wagener *et al.,* 2003). Despite the essential and beneficial role of this inflammatory process, some times it is directed against autologous antigens, or it does not diminish after its onset and then escalates into long term persistent inflammation (Wagener *et al.,* 2003).

A growing body of evidence has indicated the role of HO-1 in inflammation (Otterbein *et al.,* 2002; Sarady *et al.,* 2002; Attuwaybi *et al.,* 2004). It was reported that HO over expression or exposure to low concentrations of external CO inhibited the production of granulocyte macrophage colony-stimulating factor (GM-CSF), which is a potent cytokine produced in response to bacterial endotoxins (Sarady *et al.,* 2002). In another study HO-1 played an important role in protecting gut against ischaemia reperfusion injury, where HO-1 expression suppressed inflammatory responses and lessened mucosal injury (Attuwaybi *et al.,* 2004). The anti-inflammatory role of HO-1 was further supported by the findings in null mice that lack haem oxygenase enzyme as well as the only reported case of human HO-1 deficiency; in both cases there was profound inflammation that could not be attributed to infection or external exogenous factors (Poss and Tonegawa, 1997; Yachie *et al.,* 1999; Otterbein, 2002). It was suggested that CO released via HO-1 works as an antidote for these dysfunctional inflammatory conditions (Otterbein *et al.,* 2002). Exogenous administration of low concentrations of CO markedly attenuated inflammatory processes in different cellular and animal models of inflammation such as asthma, hyperoxia, endotoxic shock, xenotransplantation and ischaemia perfusion (Otterbein *et al.,* 1999; Chapman *et al.,* 2001; Sato *et al.,* 2001; Attuwaybi *et al.,* 2004; Mazzola *et al.*, 2004).

The mechanisms by which CO mediates its anti-inflammatory effects remain obscure but they may involve the mitogen-activated protein kinase (MAPK) pathway which transduces oxidative stress and inflammatory signalling (Otterbein *et al,* 2000). Although the mechanisms by which CO regulates inflammatory responses is not well understood, the conclusion is the same: CO at optimal concentrations, concentrations that are well below those that would otherwise be toxic, has cytoprotective effect (Otterbein *et al.,* 2002).

1.6.4 CO and the liver

The localization of HO-1 and HO-2 in the liver has distinct topographic distribution (Goda *et al.,* 1998). In contrast to HO-1which was detected only in Kupffer cells, HO-2 was observed in parenchymal cells, but not in Kupffer cells. Neither HO-1 nor HO-2 was detectable in hepatic satellite cells or sinusoidal endothelial cells (Goda *et al.,* 1998). HO-1 seems to play an important role in protecting liver against stress (Poss and Tonegawa, 1997; Yachie *et al.,* 1999; Morse and Sethi, 2002). Great oxidative damage was reported in the livers of rats lacking HO-1 (Poss and Tonegawa, 1997). Iron accumulation in the liver and the indications of profound endothelial damage in theone and only reported human case of HO-1 deficiency highlight the protective role of HO-1 against hepatic injury (Yachie *et al.,* 1999; Morse and Sethi, 2002). In perfused livers of endotoxaemic rats, CO generation mediated HO-1 protective effect against hepatobiliary dysfunction caused by haem overloading under stress condition (Kyokane *et al.,* 2001). Furthermore, CO generated from HO-2 in the parenchymal cells and released to the extrasinusoidal space, was suggested to play an important role in the maintenance of hepatic perfusion, as haemoglobin, the trapping agent of CO, induced marked sinosuidal constriction (Goda *et al.*, 1998). This hypothesis was supported by Suematsu and co-workers, who reported that the increase in sinosuidal constriction due to HO inhibition, was attenuated by external CO administration to isolated perfused rat liver (Suematsu *et al.*, 1995).

1.6.4 CO and the neurological system

The localization of HO-2 mRNA in the brain is essentially the same as that of sGC mRNA, and inhibition of HO activity depletes endogenous cGMP (Verma *et al.*, 1993). Therefore, it was suggested that like NO, CO might function as a neurotransmitter (Verma *et al.*, 1993). Intestinal myenteric plexus neurons also express HO-2, and targeted deletion of HO-2 gene diminished neurally evoked intestinal relaxation, an effect thought to be mediated by endogenous CO production (Zakhary *et al.*, 1997). These results further support the role of CO as a neurotransmitter (Zakhary *et al.*, 1997). CO also seems to have a role in regulating neuroendocrine functions as it decreased stimulated release of stress neuropeptides, such as oxytocin, and vasopressin from rat hypothalamus *in vitro* (Giusti-Paiva *et al.*, 2005). CO seems to have a role in regulating cerebral circulation as *in vitro, ex vivo,* and *in vivo* studies have demonstrated the ability of CO, endogenously produced or externally applied, to induce concentration-dependant vasorelaxation of piglet, dog, and porcine cerebral circulation (Leffler *et al.*, 1999; Komuro *et al.*, 2001; Jaggar *et al.*, 2002; Winestone *et al.*, 2003; Koneru and Leffler, 2004; Leffler *et al.*, 2005). The vasorelaxing properties of CO will be discussed in later sections.

1.6.5 CO and gastrointestinal tract

HO-2 is predominantly localised within the cell bodies of rat myenteric ganglia and nerve fibres coursing in parallel with intestinal inner circular muscle (Zakhary *et al.,* 1997). Targeted gene deletion of HO-2 resulted in diminishing the neurally evoked nonadrenergic noncholenergic (NANC) intestinal relaxation in rats. Therefore, it was concluded that HO-2 plays a role in mediating NANC relaxation. This effect was attributed to HO-2 derived CO (Zakhary *et al.,* 1997). Similarly, CO, endogenously produced or externally applied, was shown to have inhibitory effects on internal anal sphincter (Rattan and Chakder, 1993). CO elicited a concentration-dependant drop in the resting tension of internal anal sphincter (IAS). The direct effect of CO on internal anal sphincter was confirmed by its relaxing effect on isolated smooth muscle cels. Furthermore, HO inhibition suppressed the relaxation induced in internal sphincter by NANC nerve stimulation (Rattan and Chakder, 1993).

CO seems to play a major role as an endogenous hyperpolarizing factor in the gastrointestinal tract (Farrugia *et al.,* 2003). Targeted gene deletion of HO-2 resulted in depolarization of smooth muscle cells and abolished membrane potential, whereas exogenous CO hyperpolarized membrane potential in the canine gastrointestinal tract (Farrugia *et al.,* 2003). Recently, it has been reported that brief inhalation of low doses of CO protects swine and rodents from postoperative gastrointestinal ileus (Moore *et al.,* 2005). Additionally it protects against the development of intestinal inflammation in a model of experimental necrotizing enterocolitis (Zuckerbraun *et al.,* 2005). Similarly, HO-1 upregulation was reported to protect from several types of gastrointestinal injuries, such as colitis and sepsis (Gibbons and Farrugia 2004).

1.6.6 CO and transplantation

Expression of HO-1 by graft vasculature seems to be critical to achieve mouse to rat cardiac transplant survival (Soares *et al.,* 1998; Sato *et al.,*2001). This protective effect of HO-1 was attributed to CO generation. Both HO-1 derived CO and exogenous CO suppressed graft rejection and restored long term graft survival. The process by which CO exerts this effect might involve inhibition of platelet aggregation that facilitates vascular thrombosis and myocardial infarction (Sato *et al.,* 2001). Additional mechanism by which CO overcomes mouse to rat cardiac graft transplant rejection, might involve its ability to suppress cell apoptosis (Sato *et al.,* 2001). CO was also reported to protect against lung transplant rejection (Song *et al.,* 2003). This cytoprotective effect of CO was attributed to its anti inflammatory and anti-apoptotic properties (Song *et al.,* 2003). The protective role of CO was not confined to heart and lung, as CO also prolonged the survival of liver and small intestinal graft (Ke *et al.,* 2002; Nakao *et al.,* 2003a; Kaizu *et al.*, 2005).

1.7 CO and vascular functions

1.7.1 General structure of blood vessels

The general structure of arteries, veins, and arterioles is similar (Orallo *et al.,* 1996). The blood vessel is an active, integrated organ composed of various cell types that are arranged in a complex set of autocrine-paracrine interactions (Durante *et al.,* 2002). The vessel wall is organised in three distinct layers (Orallo *et al.,* 1996; Durante *et al.,* 2002). The innermost, is a layer of endothelial cells (the tunica intima), which lines the internal surface of the vessels and is seated on a specialised extracellular matrix known as the basement membrane. The underlying media (tunica media) is composed of vascular smooth muscle cells (SMCs) (20-60 μm in length and about 4 μm in diameter) that are densely packed into an interstitial matrix containing collagen, fibronectin and proteoglycans. Each smooth muscle cell extends more or less circularly around the lumen of the vessel, so when they contract or relax they cause a decrease or increase in the vessel diameter respectively (Orallo *et al.,* 1996; Durante *et al.,* 2002). In veins, VSMs can also extend longitudinally. The outer layer (adventitial layer), comprises collagen fibres, enfolded around the tunica media. The neural axons that innervate blood vessels are usually confined to this layer and rarely penetrate the tunica media (Orallo *et al.,* 1996; Durante *et al.,* 2002).

1.7.2 Endogenous production of CO from vascular tissue

It seems that HO-2 is the predominant form of haem oxygenase enzymes in the endothelium and vascular SMCs under normal conditions (Ewing *et al.,* 1994; Grozdanovic and Gossrau, 1996; Zakhary *et al.,* 1996; Maines *et al.,* 1997). It is also localised in adventitial nerves of blood vessels and carotid body chemoreceptors (Prabhakar *et al.,* 1995; Zakhary *et al.,* 1996) but not in striated cardiac musculature (Grozdanovic and Gossrau, 1996). HO-1, which is minimally expressed in vascular tissue under physiological conditions, is highly induced in vascular endothelium, SMCs and myocytes as response to different stimuli (Maines *et al.,* 1997; Foresti *et al.,* 2002). These stimuli include hypoxia (Morita *et al.,* 1995), haemorrhagic shock (Moncure *et al.,* 2003) , cAMP (Durante *et al.,* 1997), nitric oxide (NO) (Motterlini *et al.,* 1996; Hartsfield *et al.,* 1997), endotoxins (Yet *et al.,* 1997), mildly oxidized low density lipoproteins (Ishikawa *et al.,* 1997) and hemodynamic forces such as shear stress (Wagner *et al.,* 1995;Wagner *et al.,* 1997).

Many techniques have been introduced to measure the endogenous formation of CO in blood vessels (Marks *et al.,* 2002). Among these techniques, measurement of CO production using an ultrasensitive laser sensor remains one of the most sensitive because it has higher sensitivity to low CO levels and does not require chemical mediators (Morimoto *et al.,* 2001; Marks *et al.,* 2002). In this protocol quantification of CO generated in cultured rat aortic SMCs was done using an ultrasensitive laser sensor without interference from other gases (Morimoto *et al.,* 2001). The basal CO production was 0.25 ± 0.07 nmol per 10^7 cells/h and that level was not affected by treating the cells with sodium nitroprusside, a nitric oxide donor, despite marked elevation of HO-1 protein expression (Morimoto *et al.,* 2001). In contrast, treatment of the cells with hemin, the haem oxygenase substrate, resulted in around a two times increase in CO production. The hemin-induced CO synthesis was attributed to its ability to stimulate higher levels of HO-1 protein compared to sodium nitroprusside (Morimoto *et al.,* 2001). In addition, hemin provides SMCs with additional substrate for HO-1 metabolism. Interestingly, treatment of the cells with a combination of hemin and sodium nitroprusside resulted in a further increase of CO production (Morimoto *et al.,* 2001). In the same study adding tin protoporphyrin-IX (SnPP), which is a potent HO inhibitor, to hemin treated cells resulted in almost complete block of endogenous CO production.

The facts that CO is generated in blood vessels under physiological conditions and that real time endogenous CO formation in vascular tissues is measurable satisfy important criteria for a physiological role of CO in blood vessels (Marks *et al.,* 2002 a).

1.7.3 CO and vascular SMC growth

The effect of HO-1 on cell proliferation is highly variable and seems to be cell specific (Durante *et al.,* 2003). In contrast to its proliferative effect on epidermal keratinocytes and tumour cells, HO-1 appears to play an anti-proliferative role in renal and pulmonary epithelial cell and vascular SMCs (Durante *et al.,* 2003). Several studies have demonstrated the anti-proliferative effect of HO-1/ CO system on vascular SMCs (Morita *et al.,* 1997; Togane *et al.,* 2000; Peyton *et al.,* 2002). Vascular SMCs proliferation was inhibited or augmented by HO inducers or inhibitor respectively (Togane *et al.,* 2000). Haemoglobin, a reagent trapping both NO and CO, but not methaemoglobin, which can capture NO but not CO, augmented the proliferative response of rat vascular smooth muscle cells to angiotensin II (Togane *et al.,* 2000). Serum was reported to induce HO-1 gene expression in vascular SMCs, where HO-1 catalyzed CO formation in a negative feedback manner to limit serum-stimulated SMC proliferation (Peyton *et al.,* 2002). Vascular SMC derived CO has been suggested to play an important role in regulating vascular SMC proliferation under hypoxic conditions (Morita *et al.,* 1997). Inhibition of CO generation or scavenging of CO with haemoglobin, enhanced vascular SMCs proliferation in response to serum or mitogens, while increasing CO formation or exposing cells to exogenous CO resulted in significant suppression of growth response (Morita *et al.,* 1997). In addition to direct attenuation of cell proliferation, CO might regulate SMC growth by inhibiting the endothelium-released platelet derived growth factor (PDGF) or endothelin-1 (ET-1), which promotes SMCs proliferation (Morita and Kourembanas, 1996).

The influence of CO on SMC apoptosis is controversial (Durante *et al.,* 2002; Liu *et al,* 2002; Liu *et al.,* 2003). Apoptosis is programmed cell death serving many physiological functions (Allen and Agrawal, 1997). In contrast to classic cellular swelling and membrane rupture associated with necrosis, apoptotic cells shrink and maintain their membrane integrity (Allen and Agrawal, 1997). HO-1 over expression in cultured aortic SMCs induced apoptotic cell death, and incubating the vascular SMC with haemoglobin, the CO scavenger, or SnPP, the HO inhibitor, significantly reduced apoptosis (Durante *et al.*, 2002). On the other hand CO, either administered exogenously or formed endogenously, inhibited cultured aortic SMCs apoptosis (Liu *et al.*, 2002; Liu *et al.*, 2003). Clearly, more work is still needed to explore the role of CO in regulation of vascular SMC apoptosis (Durante *et al.*, 2002).

1.8 CO-Induced Vasorelaxation

Although the endogenous production of CO has been known for more than a half century, the resurgent interest in the physiological effects of CO has only really been highlighted over the last two decades (Zhao, 2002). The purification and identification of the two different HO isozymes was a turning point in the history of the haem oxygenase-carbon monoxide system (Maines *et al.*, 1986). Over the last two decades CO, endogenously produced or externally applied, has been shown to attenuate vasoconstriction and induce vasorelaxation of diverse blood vessels with different diameters and from different species possibly through different mechanisms. The affected vascular tissues include: rat and rabbit aorta (Lin and McGrath, 1988; Furchgott and Jothianandan, 1991; Brian *et al.*, 1994; Hussain *et al.*, 1997); piglet and rat pulmonary arteries and veins (Villamor *et al.*, 2000; Ding *et al.*, 2002); lamb ductus arteriosus (Coceani *et al.*, 1988; Coceani *et al.*, 1996); rat, dog and porcine coronary arteries and veins (McFaul and McGrath 1987; Vedernikov *et al.*, 1989; Graser *et al.*, 1990; Barbe *et al.*, 2002c); hepatic vein (Pannen and Bauer 1998); dog femoral and carotid artery (Vedernikov *et al.*, 1989); rat gracillus muscle arterioles (Kozma *et al.*, 1999); rat and newborn piglet mesenteric artery (Villamor *et al.*, 2000; Gonzales and Walker 2002; Naik and

Walker 2003); piglet, dog and porcine cerebral arteries and arterioles (Leffler *et al.,* 1999; Komuro *et al.,* 2001; Jaggar *et al.,* 2002; Winestone *et al.,* 2003; Leffler *et al.,* 2005); and human placental blood vessels (Bainbridge *et al.,* 2002). CO has the ability to dilate blood vessels contracted by different vasoconstrictors such as phenylephrine (adrenoreceptor agonist), U-46619 (thromboxane A_2 mimetic), methoxamine (alpha agonist), indomethacine, potassium chloride (KCl) and prostaglandin F (2 alpha) (Lin and McGrath 1988; Graser *et al.,* 1990; Coceani *et al.,* 1996; Wang *et al.,* 1997a; Komuro *et al.,* 2001; Nakao *et al.,* 2003b; Tammaro *et al.,* 2005).

As examples of its vascular activity, exogenously applied CO elicited endothelium independent vasorelaxation of rat tail arteries precontracted with phenylephrine (1 μM) (Wang *et al.,* 1997a). The CO-induced relaxation was concentration-dependant with a detectable threshold concentration of 1 μM. At 300 μM CO induced around 60 % relaxation, and 80% vasorelaxation was achieved at 1 mM. The effect of CO was sustained, but reversible upon the removal of CO from the system (Wang *et al.,* 1997a). In a model of isolated perfused rat heart, coronary flow was increased by exogenously applied CO (McFaul and McGrath, 1987). CO-induced vasorelaxation was not the result of change of heart rate or decreased oxygen content in the perfusate and was not mediated by adenosine, prostaglandin or adrenergic influence (McFaul and McGrath, 1987). The vasodilatory effect of CO on coronary vasculature was reversible as coronary flow returned to normal once CO was removed from the system (McFaul and McGrath, 1987). In an isolated ring preparation, endogenously produced CO has been reported to play a major role in regulating vascular tone in aortic arteries expressing high levels of HO-1 (Sammut *et al.,* 1998). In addition, up regulation of HO-1/CO in aortic rings largely suppressed the contractile response to phenylephrine. Exogenously applied CO also induced dose dependent, endothelium-independent vasorelaxation of isolated aortic rings but this effect was much less potent than that induced by NO (Lin and McGrath, 1988; Furchgott and Jothianandan, 1991; Brian *et al.,* 1994). In mesenteric arteries endogenous CO appears to play a significant role in attenuating the vasoconstrictor reactivity after chronic hypoxia (Gonzales and Walker, 2002; Naik and Walker, 2003). It was reported that haem-L-lysine (HHL), an HO substrate, elicited a dose-dependent vasodilatory response and reduced the vasoconstrictor sensitivity to increasing concentrations of phenylephrine in mesenteric arteries that were exposed to 48 hr chronic hypoxia (Gonzales and Walker, 2002; Naik and Walker, 2003). The effect of HHL was attributed merely to CO as the role of free iron and biliverdin as mediators of this effect was ruled out. In addition, exogenous CO produced a dose-dependant reduction in vascular tone (Gonzales and Walker, 2002; Naik and Walker, 2003).

The role of CO in regulating cerebral circulation was questioned by Brian and colleagues who reported that CO even at a high concentration (300 μM) had no significant effect on rabbit cerebral arteries whereas it induced vasorelaxation in aortic arteries (Brian *et al.,* 1994). In the same study NO elicited 80 % to 100 % relaxation of all examined cerebral arteries (Brian *et al.,* 1994). On the other hand, Leffler and co-workers reported that CO produced significant dose-dependant vasodilatation at concentrations as low as 10^{-11} M in newborn pig small cerebral arterioles (<60 μm) and at 10^{-10} M in large arterioles (>60 μM) with maximal dilatation reached by 10^{-9} M in vessels of both diameters (Leffler *et al.,* 1999). The vasorelaxing properties of CO in that study were comparable to those induced by the NO donor, sodium nitroprusside (Leffler *et al.,* 1999). These authors cited four reasons to explain the discrepancy between their results and that obtained by Brian's group: firstly, the diameters of blood vessels examined were different as they used small arterioles compared to major cerebral arteries used by the others. Secondly, their study was conducted on intact brain *in vivo*, whereas the former researchers used isolated, precontracted artery rings. Thirdly, they used newborn animals in their experiments compared to adults used by Brian and co-workers. Finally, different species were used by both studies (Leffler *et al.,* 1999). The failure of high concentrations of CO to induce vasorelaxation in large cerebral arteries provoked another research group, to re-examine the response of large cerebral arteries to CO using a similar experimental model to that used by Brian (Komuro *et al.,* 2001). Surprisingly, CO caused a concentration-dependent relaxation starting with a concentration of 57 μM. The authors of this study attributed the failure of cerebral arteries to respond to CO in the former study to the presence of carboxygen (95 % O_2 : 5 % CO_2) in their organic bath which might have reduced more than 99 % of CO from the solution so that the amount of CO in the solution was less than the investigator expected (Komuro *et al.,* 2001). Since this debate started, a growing number of articles have reported the role of endogenously produced or externally applied CO in attenuating vasoconstrictor responses and inducing vasodilatation in cerebral circulation (Jaggar *et al.,* 2002; Winestone *et al.,* 2003; Koneru and Leffler 2004; Leffler *et al.,* 2005)

It is well known that the vasodilatory potency of CO is not universal in all vascular tissues and it varies between different tissue types, species, developmental age and the diameter of the vessels (Wang *et al.,* 1998; Zhang *et al.,* 2001b; Zhao W. 2002). For example, endogenous CO acts to maintain portal venous vascular tone but has no effect on hepatic arteries (Pannen and Bauer, 1998). Another example of the tissue specificity of CO was demonstrated by experiments conducted on systemic and pulmonary vessels of newborn piglets (Villamor *et al.,* 2000). In that study, CO-induced endothelium-independent vasorelaxation of pulmonary arteries, pulmonary veins and mesenteric arteries, but that relaxation was more marked in pulmonary veins than in pulmonary arteries or mesenteric arteries. Furthermore, the vascular potency of CO in piglets arteries decreases with age as CO-induced vasorelaxation was greater in pulmonary arteries in the 12-24h old than that of 2 week old piglets. In the same study, vasorelaxation induced by NO was also greater in pulmonary veins than in pulmonary arteries; however, its potency increased with postnatal age (Villamor *et al.,* 2000). Similarly, exogenous CO which induced 76 % relaxation of pigs urethral preparations and 86 % of lower gastroesophagastric junction, failed to induce vasorelaxation of extramural arterial rings in the same study (Werkstrom *et al.,* 1997).

Despite a large number of studies demonstrating the vasorelaxing properties of CO, it was reported that CO promoted endothelium-dependant constriction of isolated gracilis muscle arterioles (Johnson and Johnson, 2003). In addition, CO contributed to salt-induced hypertension in Dahl salt sensitive rats (Teran *et al.,* 2005). In another study conducted on rat isolated gracilis arterioles, CO induced both vasodilatation and vasoconstriction (Johnson *et al.,* 1999). In the presence of intact endothelium, CO elicited vasoconstriction whereas in endothelium denuded arterioles CO elicited vasodilatation. The authors attributed this conflicting effect of CO in the same blood vessels to the effect of CO on endothelium NO. They suggested that in the presence of intact endothelium, CO might inhibit the endogenous production of endothelium NO, which is one of main regulators of vascular tone, and as a result induces vasoconstriction. Therefore, in the absence of an endothelial layer or in the presence of NO synthase inhibitors, CO was devoted to its local vasodilatory action on vascular SMC (Johnson *et al.*, 1999). This conclusion contrasts with many studies which have shown that the CO has relaxing effects on both endothelium intact and endothelium denuded vessels (Wang *et al.*, 1997a; Villamor *et al.*, 2000; Ding *et al.* 2002; Naik *et al.*, 2003). The complex architecture of vascular tissue might explain the diverse effect of CO on vascular tone reported in the literature (Archer *et al.*, 1996; Zhao *et al.*, 2002). It has been reported that the prevalence of various vascular smooth muscle populations differs from segment to segment within a single artery and also may vary in the vascular tree of different organs (Archer *et al.*, 1996). Furthermore, the composition of arterial media is plastic and changes at different developmental stages and in response to injury (Archer *et al.*, 1996). Therefore, it has been hypothesised that certain vascular tissues are equipped with different signalling mechanisms and the expression of various cellular targets of CO in these tissues may have different profiles (Zhao *et al.*, 2002).

1.9 The Mechanisms for CO-induced Vasorelaxation

A number of intracellular pathways have been proposed as mediators of CO-induced vasorelaxation; however activation of soluble guanylate cyclase (sGC), and potassium (K^+) channels, remain the two most popular mechanisms for many scientists. Therefore in the following sections detailed analysis of the possible role of these pathways in amplifying the biological effect of CO will be presented.

1.10 Soluble Guanylate Cyclase (sGC)

The intracellular second messengers involved in SMC relaxation are cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) (Carvajal *et al.,* 2000). cGMP exerts its action through activation of cGMP-dependent protein kinase (PKG) whereas cAMP elicits its action through cAMP-dependant protein kinase (PKA). For a long time cAMP was regarded as the principal second messenger involved in vasorelaxation but recent evidence points towards a primary role of cGMP that exceeds that of cAMP in relaxation of SMC by endogenous or exogenous compounds (Carvajal *et al.,* 2000). In smooth muscle cells, cGMP is generated from guanosine triphosphate (GTP) by the activity of guanylate cyclase (GC) enzyme, and degraded by cyclic nucleotide phosphodiestarase (PDE) (Carvajal *et al.,* 2000; Pyriochou and Papapetropoulos, 2005). There are two isoforms of GC; the cytosolic soluble GC (sGC) and the membrane bound particulate GC (pGC) (**Figure 1.5**). They differ in their structure, ligands and subcellular organization (Pyriochou and Papapetropoulos, 2005). Regardless of whether cGMP is formed by means of the pGC or sGC pathway, it elicits its effect by binding and modifying the activity of three types of intracellular receptors: cGMP dependant protein kinase (PKG), cGMP-binding phosphodiestarases, cGMP-regulated ion channels (Carvajal *et al.,* 2000). PKG has been considered the principle mediator of cGMP-induced vasorelaxation whereas the role of the other two receptors in cGMP-induced relaxation has not been evaluated (Carvajal *et al.,* 2000). There are two mechanisms by which cGMP might induce vasorelaxation; either by lowering cytosolic Ca^{+2} ($[Ca^{+2}]_i$) levels or reducing the sensitivity of contractile systems to Ca^{+2} (Carvajal *et al.,* 2000; Cary and Marletta, 2001). Although the mechanisms by which cGMP reduce cytosolic Ca^{+2} are complex and not well understood, an important role of PKG in mediating this action has been proposed (Lincoln *et al.,* 1994; Carvajal *et al.,* 2000). PKG has been reported to regulate several pathways that control cytosolic Ca^{+2} levels such as inhibition of inositol triphosphate formation and receptor activity, activation of Ca^{+2} activated K^{+} channels (K_{Ca}), and activation of Ca^{+2}-ATPase pumps in the sarcoplasmic reticulum and plasma membrane (Lincoln *et al.,* 1994; Carvajal *et al.,* 2000; Cary and Marletta, 2001). The physiological consequence of a transient fall in cytosolic Ca^{+2} differs depending on cell types, but in SMCs, a decline in cytosolic Ca^{+2} results in relaxation and a rise causes contraction (Cary and Marletta, 2001).

In addition, cGMP, possibly through PKG, reduces the sensitivity of the contractile system to Ca^{+2}; as a result SMCs contraction is not triggered even by supra-threshold levels of cytosolic Ca^{+2} (Carvajal *et al.,* 2000).

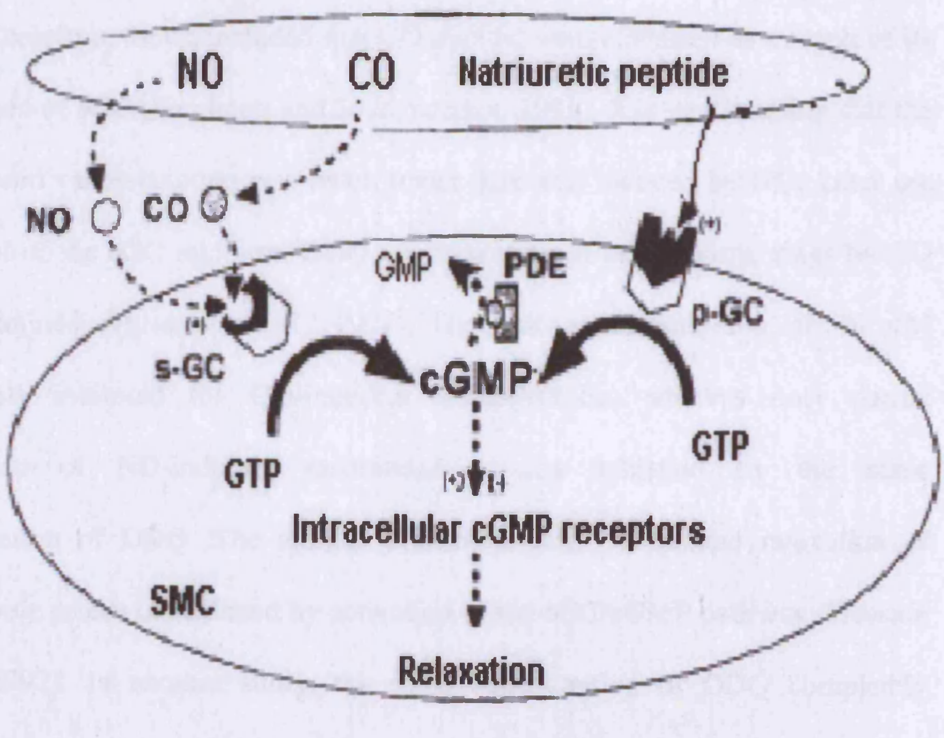


Figure 1.5. cGMP generation and action (adapted from Carvajal *et al.*, 2000).

1.10.1 CO and sGC/cGMP pathway

More than fifteen years ago, the possible role of the sGC/cGMP pathway as a mediator for the effect of CO was suggested, but it was in platelets rather than in blood vessels (Brune and Ullrich, 1988). Later on, several studies established the role of sGC as a mediator of CO-induced vasorelaxation (Wang *et al.,* 1998; Morse *et al.,* 2002; Nakatsu *et al.,* 2002; Ndisang *et al.,* 2004). Isolated blood vessel preparations were used in some laboratories to assess the vasorelaxing effect of CO and measure the effects of sGC pathway inhibitors such as methylene blue and 1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-One (ODQ) on CO-induced vasorelaxation. The direct stimulating effect of CO on cGMP production in vascular tissue and cultured vascular cells was also investigated. In the early1990s, Furchgott and co-workers reported that CO produced endothelium independent vasorelaxation of rabbit aortic rings (Furchgott and Jothianandan, 1991). That CO-induced vasorelaxation was associated with an increase in cGMP levels. Therefore, they concluded that CO elicited vasorelaxation as a result of its stimulation of sGC (Furchgott and Jothianandan, 1991). It is worth noting that the CO-induced vasorelaxation was much lower than that induced by NO. Later on, the effect of the sGC inhibitor, ODQ, on relaxation of rabbit aortic rings by CO was examined (Hussain *et al.,* 1997). ODQ at a concentration of 10 μM completely inhibited the CO-induced vasorelaxation, whereas only partial attenuation of NO-induced vasorelaxation was achieved by the same concentration of ODQ. The authors concluded that CO-elicited relaxation of rabbit aortic artery is mediated by activation of the sGC/cGMP pathway (Hussain *et al.,* 1997). In another study, the same concentration of ODQ completely inhibited the vasorelaxing effects of both CO and NO on mesenteric artery and pulmonary artery and vein of newborn piglets (Villamor *et al.,* 2000). Interestingly, the age dependant change in the effect of CO on pulmonary artery, mentioned in a previous section (page 47), was attributed to increase in phosphodiesterase, the cGMP degrading enzyme, activity during the first days of extrauterine life (Villamor *et al.,* 2000). It was reported that CO, endogenously generated from HO-1 over expression or exogenously applied, substantially increased cGMP production in an aortic ring extract (Sammut *et al.,* 1998). Another piece of evidence for the role of cGMP was provided by Motterlini's group, who investigated the effect of HO-1 expression on blood pressure *in vivo* (Motterlini *et al.,* 1998). They observed that infusion of vasoconstrictor agents that caused acute and significant elevation in rat blood pressure at five days after catheter implantation, failed to induce any change when introduced one day after surgery (Motterlini *et al.,* 1998). The suppression of a hypertensive response observed one day after surgery was associated with significant expression of HO-1 in aorta, liver and heart as well as a rise in CO formation and cGMP levels. Pre-treatment of the rats with HO inhibitors significantly lowered CO and cGMP levels and totally restored the acute hypertensive response (Motterlini *et al.,* 1998). In another study Morita and co-workers showed that CO derived from vascular SMC has paracrine effects on endothelial cells under hypoxic conditions (Morita and Kourembanas 1996). It caused a fourfold increase in endothelium cGMP formation, and inhibition of HO production or scavenging CO prevented the rise in cGMP production (Morita and Kourembanas 1996). Hypoxia, which has a profound effect on vessel tone, significantly increased HO-1 expression and activity in rat aortic and pulmonary artery smooth muscle cells (Morita *et al.,* 1995). This rise in HO-1 production was associated with similar elevation of cGMP levels and this elevation of cGMP levels was completely inhibited in the presence of tin protoporphyrin, an HO inhibitor, or haemoglobin, a scavenger of CO. In contrast, NO had no effect on cGMP production. Taken together these results show that CO may regulate vascular tone under physiological and pathological conditions, such as hypoxia, and this effect is largely mediated through cGMP activation (Morita *et al.,* 1995).

On the other hand, Suematsu and colleagues, who studied the role of endogenously produced CO in modulating sinusoidal tone in liver perfusate, hypothesised that CO-induced vasorelaxation in sinusoids was possibly due to cGMP-independent mechanisms (Suematsu *et al.,* 1995). Two main findings supported their hypothesis: firstly, the minimum concentration of CO which was needed to induce a significant rise in cGMP levels in cultured Ito cells was at least 100 times higher than that detected in the effluent of the control perfusate liver. Secondly, 8-bromo-cGMP, the cGMP analogue, reversed only 60 % of vasoconstriction induced by the HO inhibitor Zn PP. Therefore, they suggested a possible role of potassium channels (K^+) in mediating the vasorelaxing properties of CO (Suematsu *et al.,* 1995). Cerebral circulation has provided other examples of the controversy about the role of cGMP in mediating the vasorelaxing properties of CO. It was reported that piglet's pial arteriolar dilatation to CO was completely reversed by ODQ but CO did not increase cGMP. And because the minimal level of cGMP analogue restored the vasorelaxing effect of CO after inhibition with ODQ, it was concluded that a certain threshold level of cGMP is necessary to allow CO to increase K_{Ca} channel activity to induce dilatation (Koneru and Leffler, 2004). In dog cerebral artery segments, the CO-induced relaxation was reduced in the presence of ODQ or TEA, the non selective K^+

inhibitor. Neither ODQ nor TEA completely inhibited the vasorelaxing effect of CO and there was no additive effect when ODQ and TEA were administered together (Komuro *et al.,* 2001). The role of cGMP in mediating the vasorelaxing properties of CO in mesenteric arterioles varies according to the source of CO (Naik and Walker, 2003). Both HO derived and exogenous CO elicited concentration dependent vasorelaxation of chronically hypoxic superior mesenteric arterioles. However, endogenous CO-induced vasorelaxation was not altered in the presence of ODQ whereas the vasodilatation induced by external CO was almost completely reversed by the same concentration of ODQ. Therefore it was suggested that the vasodilatory response of superior mesenteric arterioles to endogenous CO is possibly mediated by cGMP independent mechanisms (Naik and Walker, 2003). Similarly, Coceani and colleagues, who investigated the effect of CO on lamb ductus arteriosus concluded that the primary action of CO in ductus arteriosus is not mediated by sGC/cGMP pathway and that the sGC/cGMP pathway may only have an accessory role in the relaxation to CO (Coceani *et al.,* 1996). Instead they emphasized the importance of cytochrome P450-based mono-oxygenase reaction as a target for CO activity in the lamb ductus arteriosus (Coceani *et al.,* 1996).

In summary, it appears that cGMP plays an important role in mediating the vasorelaxing properties of CO on at least some blood vessels (Nakatsu *et al.,* 2002). The correlation between CO and cGMP levels seems to be clearer in larger vessels than smaller vessels, which tend to be more independent of cGMP (Nakatsu *et al.,* 2002).

1.10.2 Benzyl indazole derivative (YC-1) and CO

The degree of purified sGC enzyme activation *in vitro* differs significantly in response to NO and CO (Stone and Marletta, 1994; Friebe *et al.,* 1996). It has been shown that CO induced about a 4-5-fold rise in sGC activity while NO induces a 100- 400-fold increase (Stone and Marletta, 1994; Friebe *et al.,* 1996). In addition, several studies have documented that CO has lower potency compared to NO in relaxing blood vessels (Furchgott and Jothianandan, 1991; Brian *et al.,* 1994). Therefore, the hypothesis that endogenous CO can play a role similar to that of NO in regulating vascular tone has been questioned (Brian *et al.,* 1994; Hartsfield *et al.,* 2002; Nakatsu *et al.,* 2002). However, these criticisms have been muted by the demonstration that in the presence of YC-1, CO is capable of stimulating purified sGC to a degree similar to NO (Friebe *et al.,* 1996; Stone and Marletta, 1998). YC-1 is an NO and CO-independent activator of sGC activity (Friebe *et al.,* 1996). It induced around 12-fold increase in purified sGC activity. In the presence of YC-1, the stimulatory effect of CO was potentiated enormously, and CO was capable of stimulating sGC 106-fold which is similar to levels induced by NO (Friebe *et al.,* 1996). Consistent with these results, it was reported that YC-1 potentiated CO-induced relaxation of rat aortic strips by around 10 times (McLaughlin *et al.,* 2000). Therefore McLaughlin and colleagues hypothesised that should an endogenous compound exist with similar properties to that of YC-1, then the vasoactivity of CO in the presence of this factor would be increased, and CO could have a role in the regulation of vascular tone comparable to that of NO (McLaughlin *et al.,* 2000). This conclusion has been shared by Friebe and colleagues, who proposed that the existence of an endogenous YC-1-like substance that is expressed in a cell or tissue specific manner might explain the controversy about the role of cGMP in mediating the vasorelaxing properties of CO. Depending on the presence or absence of such an endogenous modulator, CO would display high or low level sGC-activating properties (Friebe *et al.,* 1996).

1.11 CO and K^+ Channels

An introduction to potassium (K^+) channels physiology will be presented in the next section followed by thorough analysis of the interaction between CO and K^+ channels.

1.11.1 K^+ channels

K^+ channels are groups of integral membrane proteins that selectively transport K^+ ions across the cell membrane (Korn and Trapani, 2005). They have been found in all mammalian cells and have diverse functions in both excitable and non excitable cells. In fact K^+ channels are more sophisticated than any other ion channel super family found in the mammalian cells (Cao *et al.,* 2002). In arterial smooth muscle cells almost every physiological vasoconstrictor and vasodilator has been shown to modulate one type or another of K^+ channels (Standen and Quayle, 1998). In general most vasoconstrictors and vasodilators have multiple pathways of action and, among these pathways; K^+ channels contribute to regulate membrane potential and vascular contractile tone (Standen and Quayle, 1998). It is well known that activation of K^+ channels in arterial smooth muscle cells (SMCs) induces vasodilatation and lowers blood pressure, whereas inhibition of these channels leads to vasoconstriction (**Figure 1.6**) (Nelson *et al.,* 1990; Sobey *et al.,* 2001). At least four K^+ channel subgroups have been identified in vascular SMCs (Nelson and Quayle, 1995; Standen and Quayle, 1998; Cao *et al.,* 2002; Korovkina and England, 2002b; Jackson *et al.,* 2005): The first one is calcium (Ca^{2+}) activated K^+ channels (K_{Ca}) which respond to changes in intracellular Ca^{2+} to regulate intrinsic tone in small arteries (Brayden and Nelson, 1992). It has been proposed that any rise in intravascular pressure, through cell membrane depolarization and elevation of intracellular Ca^{2+} activates K_{Ca} channels. Activation of K_{Ca} channels leads to K^+ efflux, which counteracts membrane depolarization and vasoconstriction (Brayden and Nelson, 1992). K_{Ca} channels are divided into three groups: big conductance (BK_{Ca}), intermediate conductance (IK_{Ca}) and small conductance (SK_{Ca}) channels (Brayden and Nelson, 1992). The second K^+ channel subgroup is voltage-dependant K^+ (Kv) channels which respond to depolarization stimuli (Cao *et al.,* 2002). Activation of Kv channels causes efflux of K^+ ions, which leads to membrane hyperpolarization and maintains the resting membrane potential at -40 to - 60 mV under physiological conditions (Cao *et al.,* 2002). The third subgroup is inward rectifier K^+ (Kir) channels which appear to mediate external K^+-induced hyper polarization and dilatation of resistance arteries (Nelson and Quayle, 1995). Finally the fourth subgroup is ATP sensitive K^+ (K_{ATP}) channels which is sensitive to some molecules produced from cellular energy metabolism (Zhuo *et al.,* 2005). Among these molecules, adenosine triphosphate (ATP) and adenosine diophosphate (ADP) are the most common. The decrease of intracellular ATP leads to opening of the K_{ATP} channels, which will induce vasodilatation and increase blood flow into organs (Zhuo *et al.,* 2005).

BKca and Kv channels are the most prevalent K^+ in vascular SMCs (Korovkina and England, 2002a; Korovkina and England, 2002b). In arterial smooth muscle cells which contain Kir, K_V, K_{Ca}, and K_{ATP} channels, the approximate number of channels per cell range from around 100-500 per cell for Kir and K_{ATP}, up to 1000-10000 K_{Ca}, and K_V channels per cell (Nelson and Quayle, 1995).

The role of K^+ channels in endothelial cells (EC) is not as well understood as that in SMCs (Coleman *et al.,* 2004). Kca, Kv, K_{ATP} and Kir channels have all been identified in endothelial cells (Coleman *et al.,* 2004). In contrast to SMCs, IKca and SKca are abundant in EC and it appears that these channels play essential roles in production of endothelium-derived hyperpolarizing factor (EDHF) which travels through myoendothelial gap junctions to SMCs to induce hyperpolarization and vasorelaxation (Coleman *et al.,* 2004).

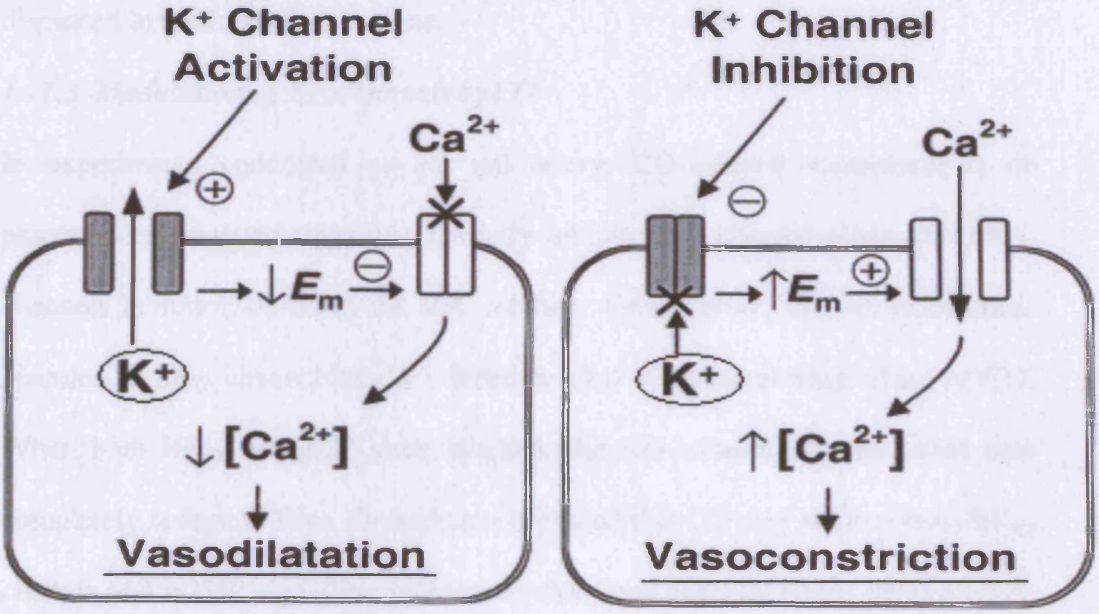


Figure 1.6. Schematic illustration of the key events involved in the vascular smooth muscle response to K^+ channel activation (left) or inhibition (right) (adapted from Sobey *et al.,* 2001).

1.11.2 Interaction between CO and K^+ channels

Ten years ago Suematsu and co-workers, hypothesised that cGMP-independent mechanisms might be involved in mediating the CO-induced vasorelaxation in hepatic sinusoids (Suematsu *et al.,* 1995). They anticipated that K^+ channels were possible cellular targets for CO in hepatic microcirculation and supported their view by a number of studies which demonstrated the interaction between CO and

K^+ in non vascular tissue such as corneal epithelial and intestinal smooth muscle cells (Rattan and Chakder, 1993; Rich *et al.,* 1994; Suematsu *et al.,* 1995). Later on, several studies have demonstrated the involvement of K^+ channels subgroups in mediating CO-induced vasorelaxation. Among these subgroups, BK_{Ca} has been considered as the most probable cellular target of CO. The role of each subgroup, especially K_{Ca} channels, in mediating the vasorelaxing effect of CO will be discussed in the following sections.

1.11.3 Modulation of K_{Ca} channels by CO

In experiments conducted on rat tail artery, CO-induced vasorelaxation of precontracted isolated rings was partially inhibited by charybdotoxin, the BK_{Ca} channels inhibitor, or ODQ, the sGC inhibitor (Wang *et al.,* 1997a). In contrast, apamine, a SK_{Ca} channel blocker, failed to alter the vasorelaxing effect of CO. When both BK_{Ca} and sGC were blocked, the CO-induced vasorelaxation was completely reversed. Thus, the authors concluded that CO may activate both BK_{Ca} chnnels and cGMP signalling pathway in the same vascular tissue (Wang *et al.,* 1997a). In another study, run by the same group, the direct effect of CO on the activity of K_{Ca} channels in isolated single SMCs of rat tail arteries was examined *in vitro* (Wang *et al.,* 1997b). At 10 μM, CO hyperpolarised isolated SMCs from -62 to -84 mV. Additionally, CO (10 μM) enhanced whole-cell outward K^+ channel currents and this was reversed quickly after removal of CO from the solution. At the same concentration, CO increased the open probability of BK_{Ca} channels by around 183 %, and this effect was antagonized by charybdotoxin but not apamine (Wang *et al.,* 1997b). Although CO did not increase the resting levels of intracellular free calcium concentration ($[Ca^{+2}]_i$), it significantly enhanced the calcium sensitivity of single BK_{Ca} channels in SMCs which correlates with increased open probability of these channels (Wang *et al.*, 1997b). The absence of GTP and cGMP dependant protein kinase in the recording solutions excluded the role of cGMP in mediating the effect of CO on BK_{Ca}. Therefore it was hypothesised that CO can directly affect BK_{Ca} by increasing its sensitivity to ($[Ca^{+2}]_i$) (Wang *et al.*, 1997b). A similar conclusion was reached by another group who investigated the effect of CO on K_{Ca} channels in newborn porcine cerebral arterioles SMCs (Xi *et al.*, 2004).

In addition to systemic circulation, BK_{Ca} has been shown to mediate CO-induced vasorelaxation in pulmonary circulation (Dubuis *et al.*, 2005). Continous inhalation of low concentrations of exogenous CO, attenuated the development of hypoxic pulmonary artery hypertension *in vivo*, and increased the sensitivity of BK_{Ca} channels to intracellular Ca^{2+} in pulmonary artery myocytes *in vitro*. The enhancement of BK_{Ca} sensitivity to Ca^{2+} increased the activity of BK_{Ca} which in turn decreased membrane depolarization of pulmonary artery myocytes induced by chronic hypoxia (Dubuis *et al.*, 2005). Because a hallmark of pulmonary artery hypertension is a sustained pulmonary artery vasoconstriction via depolarization of pulmonary artery myocytes, it was suggested that chronic inhalation of low concentrations of CO attenuates hypoxic pulmonary artery hypertension development presumably through activation of BK_{Ca} channels (Dubuis *et al.*, 2005).

K_{Ca} channels have been shown not only to mediate exogenously applied CO-induced vasorelaxation but also the endogenously produced gas (Naik and Walker 2003). HO-derived CO induced a dose dependant vasodilatory response of isolated mesenteric arterioles from chronically hypoxic rats that was nearly eliminated in the presence of iberiotoxin, the BK_{Ca} channels inhibitor, but not by the cGMP inhibitor, ODQ (Naik and Walker 2003). In addition, endogenously produced CO hyperpolarized vascular SMC membrane potential of mesenteric arteriolar strips and this effect was also inhibited by iberiotoxin. Therefore it is sensible to conclude that within mesenteric circulation, the vasodilatory response to endogenous CO involves cGMP-independent activation of BK_{Ca} (Naik and Walker, 2003).

It has been hypothesised that the gating of BK_{Ca} is under the influence of different amino acid residues, and the interaction between CO and certain amino acid residues, such as histidine, might be responsible for the activation of BK_{Ca} channels by CO (Wang and Wu, 1997; Wang *et al.,* 1998; Jaggar *et al.,* 2005). It appears that NO and CO act on different amino acid residues of BK_{Ca} channel proteins of vascular SMCs and the interaction between NO and CO might determine the functional status of BK_{Ca} channels (Wang and Wu 2003).

1.11.4 Modulation of SK_{Ca} and K_{ATP} channels by CO

Small conductance K^+ channels (SK_{Ca}) and ATP sensitive K^+ channels (K_{ATP}) have been involved in mediating the coronary vasodilatation in isolated perfused hearts of rats exposed to one week of high CO concentration (Barbe *et al.,* 2002c). CO induced vasodilatation of coronary arteries was inhibited by apamine, the inhibitor of SK_{Ca}, and glibenclamide, a K_{ATP} channel blocker, but not by methylene blue, an sGG inhibitor. In addition chronic exposure to CO induced hyperpolarization of coronary artery rings. Therefore it was concluded that CO-induced vasodilatation of coronary arteries is mediated by cGMP independent activation of SK_{Ca} and K_{ATP} (Barbe *et al.,* 2002c).

1.11.5 Modulation of K_V channels by CO

Voltage dependant K^+ channel (K_V) current was activated in coronary vascular

SMCs of rats submitted to three weeks of CO exposure (Barbe *et al.,* 2002b). Chronic exposure to CO hyperpolarized isolated coronary artery myocytes *in vitro* and enhanced coronary blood flow *ex vivo*. Although the connection between these actions has not been established, these results point toward a possible role of K_V channels in mediating CO-induced coronary vasodilatation (Barbe *et al.,* 2002b).

1.12 Other Possible Mediators of CO-induced Vasorelaxation

Inhibition of the cytochrome P-450 enzyme pathway, which produces a number of vasoconstrictor metabolites, has been presumed as one of the mechanisms by which CO mediates its vasorelaxing effects particularly in lamb ductus arteriosus (Coceani *et al.,* 1984; Coceani *et a.,* 1988; Coceani *et al.,* 1996). However, the role of cytochrome P-450 enzymes pathways in mediating CO-induced vasorelaxation was ruled out in rat aortic artery (Wang *et al.,* 1997a; Wang *et al.,* 1998).

In addition to the previous mechanisms, there are other cellular targets which might be involved in CO-induced vasorelaxation. These cellular pathways include calcium (Ca^{2+}) channels, voltage-gated Na^+ channels, Na^+/K^+ ATPase and cAMP pathway (Wang *et al.,* 1998; Zhao *et al.,* 2002). However, the importance of these mechanisms in mediating CO-induced vasorelaxation is controversial and many studies are needed to extend our understanding of their roles (Wang *et al.,* 1998; Zhao *et al.,* 2002).

1.13 Comparative Properties of CO and NO

Both NO and CO share similar properties (Hartsfield *et al.,* 2002). As gases, NO and CO have similar solubility in water (2.6 mL/100mL and 4.7 mL/100 ml at 20

^{0}C) and molecular mass (28.01 and 30.01) respectively (Foresti and Motterlini, 1999; Hartsfield *et al.,* 2002). Both NO and CO are generated from the vascular wall via the constitutive and inducible forms of NOS or HO enzymes respectively. Both induced vasorelaxation and activate sGC (Foresti and Motterlini, 1999; Hartsfield *et al.,* 2002). On the other hand, remarkable differences between CO and NO do exist. NO is a free radical and can react with oxygen derived radicals to produce toxic free radicals (Maines *et al.,* 1997; Marks *et al.,* 2002a). In contrast, CO is not a free radical and therefore cannot induce tissue damage associated with inflammation or cytotoxicity to invading pathogen that is elicited by free radical species (Maines *et al.,* 1997). NO is very labile, with a biological half life of seconds, whereas CO is very stable and highly resistant to transformation (Johnson *et al.,* 1999). Unlike NO which binds to both ferrous and ferric haemoproteins, CO binds only to ferrous haem (Durante and Schafer, 1998). Therefore, CO can work as a more stable and selective signalling molecule (Durante and Schafer, 1998).

NO, CO, and O_2 differ in their relative affinity for haem (Maines *et al.,* 1997). CO binds more firmly to haem than O_2 and easily displaces it. NO has a much higher affinity for haem than CO and replaces CO from its linkage with haem (Maines *et al.,* 1997). In addition the dissociation rate of NO from haemoglobin is slower than that of CO. Therefore the affinity of NO to haemoglobin is around 1500 times that of CO (Foresti and Motterlini, 1999). Interestingly, the affinity of CO to haem is enhanced by up to 15 times in the presence of NO (Maines *et al.,* 1997). In general, the capacity of mammalian tissue to produce CO is high, and in some organs such as the brain it is much higher than the NO-generating capacity (Maines *et al.,* 1997). Therefore CO may compete with NO for haem binding

(Maines *et al.,* 1997; Wang *et al.,* 1998).

1.13.1 Interaction between CO and NO

Although much is known about the NO and CO production pathways, how these systems interact is less understood (Hartsfield *et al.,* 2002). A number of studies have reported the inhibitory effect of HO on NO production (White and Marletta, 1992; Chakder *et al.,* 1996; Turcanu*et al.,* 1998; Cavicchi *et al.,* 2000). HO can decrease NO production by different mechanisms (Maines *et al.,* 1997). Firstly, because NOS is a haemoprotein, HO may accelerate catabolism of newly synthesised haem and impair the production of NOS. Secondly, NOS is a haemoprotein of the cytochrome P-450 type, which is a substrate for HO, hence any increase in HO activity would be expected to accelerate the turnover rate of NOS. Thirdly, both NOS and HO systems require NADPH as cofactor, and reduction of biliverdin to bilirubin also utilizes NAPDH, thus the competition would favour the HO system (Maines *et al.,* 1997). CO, at high concentration, seems to inhibit NOS activity and NO generation whereas at lower concentrations it might induce NO release from the intracellular pool (Thorup *et al.*, 1999).

NO has been shown both to reduce and enhance HO activity (Willis *et al.*, 1995; Motterlini *et al.,* 1996). It was reported that both NO donors, and NOS activation, augmented HO activity of porcine aortic endothelial cells by various degrees (Motterlini *et al.,* 1996). These findings have been supported by other studies establishing the stimulatory effect of NO and NO donors on HO production (Foresti *et al.,* 2002; Hartsfield *et al.,* 2002). On the other hand, NO donor sodium nitroprusside has been shown to reduce haem HO activity in rat brain and spleen (Willis *et al.,* 1995). Additionally, the NO donor, molsidomine attenuated the accumulation of HO-1 mRNA and protein and the rise in HO activity after haemorrhagic shock in rat liver (Hoetzel *et al.,* 2001). The mechanisms underlying the modulation of HO by NO are still unclear, and it seems that the discrepancy between studies depends on the cellular concentration of HO versus NOS, which particular HO isoforms are present, and distinct characteristics of the various NO donors used in these studies (Hartsfield *et al.*, 2002).

1.13.2 NO and CO and regulation of vascular tone

To date there is no unifying hypothesis explaining the role played by these two gases in regulating vascular tone. For example Morita and colleagues has hypothesised that, in normal vasculature, the basal production of CO might be lower than NO, and CO is a less potent vasodilator than NO (Morita and Kourembanas, 1996). Therefore NO, not CO, might be the predominant regulator of vascular integrity under basal conditions. However, once endothelial NO production is impaired for any reasons, such as hypoxia, vascular SMC-derived CO may take over as the important regulator of vascular tone (Morita and Kourembanas, 1996). This hypothesis does not fit with the speculations of Wang's group who attempted to explain the coordination between NO and CO in vascular tissue differently (Wang *et al.,* 1998). They have hypothesised that endothelium derived NO relaxes vascular SMC in a transient and fast reacting way, while SMC-derived CO exerts an autocrine and (or) paracrine vascular effect to provide long lasting effects. They have assumed that under certain physiological or pathophysiological conditions, both gases may cooperate together to coordinate the fine tuning of vascular tone. The long acting CO might set a basal level of vascular tone, which can be transiently amplified by a brief surge of NO. However under other conditions, such as hypoxia, one gas may prevail over the other (Wang *et al.,* 1998).

Foresti and co-workers have brought together these hypothesises by presuming that HO/CO and NOS/NO cooperation to maintain cellular haemostasis depends on the localization and the physiological or pathophysiological condition being considered (Foresti and Motterlini, 1999). Importantly, under certain situations, one enzymatic pathway might counter-regulate, compensate or prevail over the other (Foresti and Motterlini, 1999).

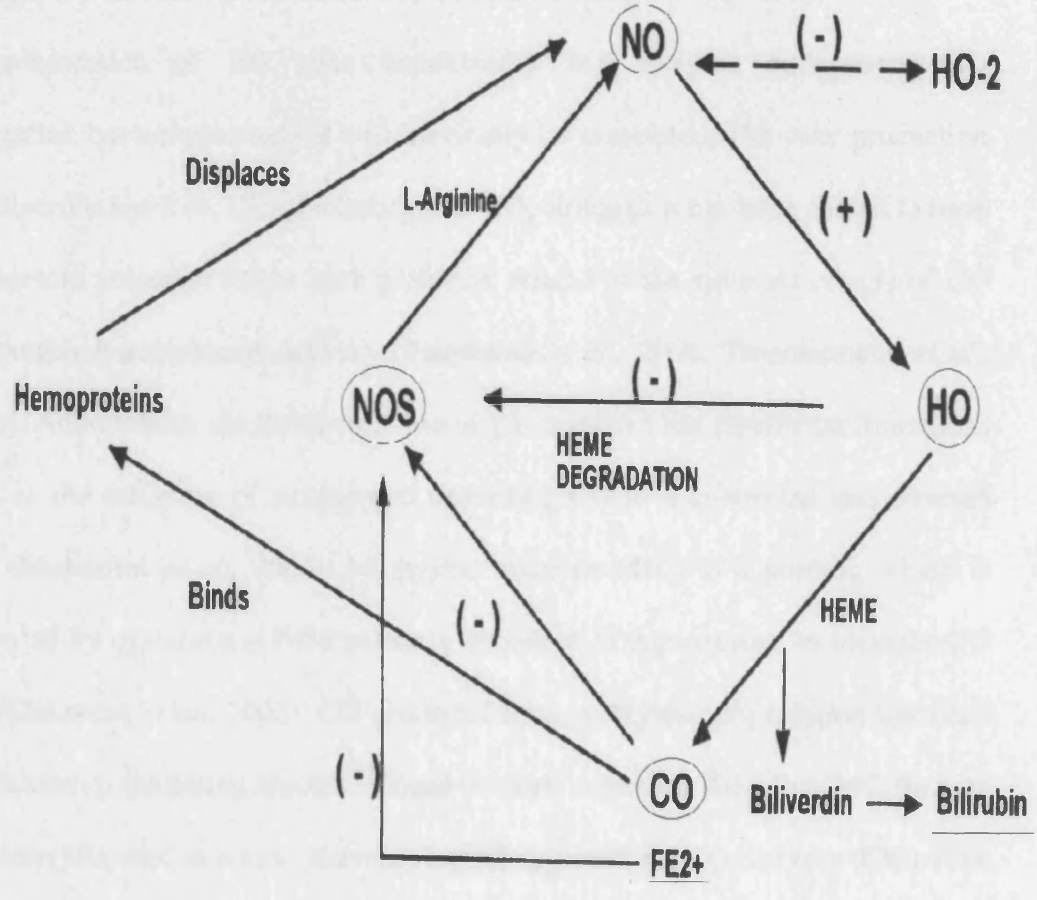


Figure 1.7. Schematic of the potential regulatory interactions between HO/CO and NOS/NO systems(adapted from Hartsfield *et al.*, 2002)

1.14 Carbon Monoxide Releasing Molecules (CO-RMs)

At present four different approaches have been proposed for examining the therapeutic potential of CO (Clark *et al.,* 2003): (1) overexpression of HO gene by targeted gene transfer (Abraham *et al.,* 2003); (2) direct administration of CO gas (Thiemermann *et al.,* 2001; Otterbein *et al.,* 2002);(3) use of prodrugs, such as methylene chloride, that are catabolized largely by hepatic enzymes to generate CO (Chauveau *et al.*, 2002); and (4) transport and delivery of CO by means of specific CO carriers (Motterlini *et al.,* 2002a; Motterlini *et al.,* 2003).

Overexpression of HO gene undoubtedly will increase endogenous CO generation but unfortunately it will inevitably be associated with over production of biliverdin and iron. Direct inhalation of CO, although it has been shown to have therapeutic potential, might have problems related to the systemic effects of CO on oxygen transport and delivery (Piantadosi *et al.,* 2002; Thiemermann *et al.,* 2001). Additionally, the therapeutic use of gas mixtures has significant limitations such as the difficulty of storing and delivering CO in a controlled and directed way (Motterlini *et al.,* 2005). Methylene chloride (MC) is a prodrug which is degraded by cytochrome P450 pathway abundant in hepatocytes, to produce CO gas (Chauveau *et al.,* 2002). CO produced from methylene degradation has been implicated in inhibiting chronic allogeneic aorta rejection, therefore MC therapy has been proposed as a new pharmacological approach for CO delivery (Chauveau *et al.,* 2002). However, this approach has significant pitfalls. Firstly, to be degraded, MC requires adequate liver function which may be compromised as is the case during haemorrhagic shock (Chauveau *et al.,* 2002; Chatterjee *et al.,* 2004). Secondly, at low concentration MC degradation appears to be carcinogenic in rodents, and at high concentration MC might have carcinogenic effect on human (Chauveau *et al.,* 2002). Thirdly, in addition to CO, MC degradation produces also CO_2 gas (Chauveau *et al.,* 2002).

It has been hypothesised that the discovery of compounds that can carry and exert measurable and safe delivery of CO to biological systems undoubtedly would facilitate the development of novel pharmaceutical agents suitable for therapeutic applications (Motterlini *et al.,* 2002a; Motterlini *et al.,* 2003). This approach could offer significant advantages over the previously mentioned approaches (Motterlini *et al.,* 2002a; Motterlini *et al.,* 2003). Therefore, our group have focused on developing molecules that are capable of carrying and delivering CO under appropriate conditions and function as CO-releasing molecules (CO-RMs). The first two compounds initially identified and possessing such prerequisites were the carbonyl complexes manganese decarbonyl ($[Mn_2(CO)_{10}]$) and tricarbonyldichloruthenium (II) dimer ($[Ru(CO)_3 Cl_2]_2$), which have been subsequently termed CORM-1 and CORM-2, respectively (Motterlini *et al.,* 2001; Motterlini *et al.*, 2003). A special myoglobin assay designed by Dr Motterlini, has proved the ability of these molecules to release measurable and controlled levels of CO (Motterlini *et al.*, 2003). Both compounds could convert deoxymyoglobin to carboxyhaemoglobin, which indicates that CO was liberated from these metal carbonyls. Furthermore, both compounds exerted effects that are similar to those mediated by CO gas, including vasorelaxation, attenuation of coronary vasoconstriction and suppression of acute hypertension (Motterlini *et al.,* 2002a; Motterlini *et al.*, 2003). However, these CO-RMs have significant drawbacks. Both are soluble only in organic solvent such as DMSO, and CORM-1 requires light to induce CO donation (Motterlini *et al.*, 2002a; Motterlini *et al.*, 2003).

Subsequently, our group made additional progress by synthesising the first prototype of a water soluble CO-RM, which was achieved primarily to overcome the solubility problems of CORM-1 and CORM-2 (Motterlini *et al.*, 2002b; Clark *et al.*, 2003; Motterlini *et al.*, 2003). This compound, tricarbonylchloro(glycinato)ruthenium(II), which has been termed CORM-3, could be obtained by coordinating the amino acid glycine onto a metal center (Motterlini *et al.*, 2002b; Clark *et al.*, 2003; Motterlini *et al.*, 2003). *In vitro, ex vivo,* and *in vivo* experimental models have demonstrated that CORM-3 can rapidly release CO in biological models (Motterlini *et al.*, 2003). Over the last few years, CORM-3 has been shown to have many beneficial effects similar to those mediated by CO in different experimental models. CORM-3 protected myocardial tissues against ischaemia reperfusion injury both *ex vivo* and *in vivo* and prolonged the survival of cardiac allografts in rats (Clark *et al.*, 2003; Guo *et al.*, 2004). Additionally, CORM-3 protected against cisplatin induced nephrotoxicity (in press) and attenuated the inflammatory response elicited by lipopolysaccaride *in vitro* (Sawle *et al.*, 2005b).

In their unrelenting search for compounds that could be safely used as CO-RMs in biological systems and eventually as therapeutic agents, our group has identified sodium boranocarbonate $Na_2[H_3BCO_2]$, later termed CORM-A1, as a new water soluble compound that can spontaneously liberate controllable and measurable amounts of CO in aqueous solution (Motterlini *et al.*, 2005). Unlike CORM-3, CORM-A1 does not contain a transition metal, and its rate of CO release, estimated by myoglobin assay, is slower than that of CORM-3. It is worth notice that our group has introduced a new amperometric CO sensor, as a novel approach for measuring CO liberated from CO-RMs. The slow pattern of CO loss from CORM-A1, estimated by myoglobin assay, was confirmed by this CO sensor (Motterlini *et al.,* 2005). The differences between CORM-3 and CORM-A1, in terms of chemical structure and kinetic of CO liberation, have tempted us to further investigate their possible impacts on the pharmacological effects of both molecules.

Later on our group has identified a new water soluble CO-releasing molecules, with different chemical structure. ($[Fe(CO)_4(\eta^3\text{-}C_3H_4Me)]BF_4$), which has been designated as CORM-319, is an iron containing metal carbonyl molecule, that can release CO spontaneously in aqueous solutions (unpublished data from our group).

We believe that the identification of new chemicals that can carry and release CO may provide significant information for optimizing therapies based on the use of this gaseous molecule. In this thesis we extend our knowledge on the bioactivity of CO-RMs in vascular tissue by analyzing the vasorelaxing and cytotoxic properties of the CO-RMs mentioned above. In addition, a detailed comparison between the possible cellular targets of CORM-A1 and CORM-3, molecules is also presented.

1.15 Hypothesis

The hypothesis being tested is whether the CO released from CORM-3, CORM-A1, and CORM-319 can induce vasorelaxation of aortic arteries, and to see whether the vasorelaxing properties of CORM-3 and CORM-A1, like that of CO gas, are mediated by activation of BK_{Ca} channels and the sGC/cGMP pathway.

1.16 AIMS

The aims of this project are:

- To study the ability of CORM-3, CORM-A1, and CORM-319, to spontaneously release CO in a safe and measured way that can induce controllable vasorelaxation in precontracted isolated aortic rings.
- To study the cellular mechanisms mediating the vasorelaxing properties of CORM-3 and CORM-A1, and to assess the effect imposed by their different pattern of CO release on their pharmacological activities.
- To assess the cytotoxic effects of CORM-3, CORM-A1, and CORM-319 on aortic artery SMCs *in vitro.*
- To investigate the effect imposed by a small difference in the chemical structure of different iron-containing CO-RMs on their vascular activity and cytotoxicity *in vitro.*

2 Materials and Methods

2.1 Synthesis of Solutions

2.1.1 Synthesis of tricarbonylchloro(glycinato) ruthenium(II) (CORM-3)

Tricarbonylchloro(glycinato)ruthenium(II) ([Ru(CO)$_3$Cl(glycinate)] or CORM-3 was synthesized as previously described (Clark *et al.*, 2003). CORM-3 was stored in closed vials at -20°C and used freshly on the day of the experiments by dissolving the compound in distilled water. Inactive CORM-3 (iCORM-3) was obtained by leaving CORM-3 in Krebs-Henseleit buffer overnight at room temperature. This treatment produced a compound that does not release CO and, therefore iCORM-3 was used as negative control for CORM-3 as already reported (Clark *et al.*, 2003). Approximately 1 mol of CO per mol of CORM-3 is liberated within 10 min after addition to Krebs Henseleit buffer (Clark *et al.*, 2003; Foresti *et al.*, 2004). Throughout our project different batches of CORM-3 with the same chemical structure but with little difference in their rate of CO release have been used.

2.1.2 Synthesis of sodium boranocarbonate ($Na_2[H_3BCO_2]$) (CORM-A1)

CORM-A1 was prepared as previously described by Alberto and co-workers (Alberto *et al.*, 2001). Stock solutions of CORM-A1 were freshly prepared before the experiments by dissolving the compounds in pure distilled water. It has been noticed that acidic pHs significantly accelerate the spontaneous release of CO from CORM-A1 (Motterlini *et al.*, 2005). We therefore took advantage of this specific property of CORM-A1 and generated an inactive form (iCORM-A1) to be used as negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N_2 through the solution for 10 min in order to remove the residual CO gas. The solution of iCORM-A1 was finally adjusted to pH=7.4 and tested with the myoglobin assay prior to experiments to verify its inability to liberate CO (Motterlini *et al.,* 2005). At pH 7.4 and room temperature the $t_{1/2}$ of CO release from CORM-A1, assessed by myoglobin assay and amperometric sensor, was 21 min (Motterlini *et al.*, 2005).

2.1.3 Synthesis of CORM-319 ([Fe(CO)$_4$(η^3-C$_3$H$_4$Me)]BF$_4$)

This compound was prepared using a method previously described (Dieter *et al.,* 1981; Enders *et al.,* 2001). A stock solution of CORM-319 was prepared freshly on the day of experiment and used within one min to preparation. When the rate of CO release from CORM-319 was assessed using myoglobin assay it was shown to be relatively slow ($t_{1/2}$ 22 min) but it was faster ($t_{1/2}$ 1-2 min) when assessed using amperometric CO sensor (unpublished data from our group). One mol of CO is approximately released by each molecule of CORM-319. CORM-319 is rapidly dissociated in distilled water (within 5 min). The inactive form (iCORM-319) was prepared by leaving the CORM-319 in distilled water overnight. This treatment produced a compound that does not release CO when assessed by myoglobin assay (unpublished data from our group).

2.1.4 Synthesis of CORM-307 ([Fe(CO)$_3$I(η^3-C$_3$H$_5$)])

CORM-307 was synthesised as previously described (Nesmeyanov *et al.,* 1968). CORM-307 was dissolved in DMSO and used freshly on the day of experiment. When assessed by myoglobin assay, CORM-307 was found to release CO very rapidly ($t_{1/2}$ ~1-2 min) and approximately one mole of CO is released by one mole of CORM-307 (unpublished data from our group). The inactive form (iCORM-307), which does not release CO and was used as negative control, was prepared in DMSO solution and left at least for 48 h to decompose. The iCORM-307 solution was finally bubbled with a stream of nitrogen to remove any residual CO present in the solution (unpublished data from our group).

2.1.5 *Synthesis of CORM-308 ($[Fe(CO)_3Br(\eta^3\text{-}C_3H_5)]$)*

CORM-308 was synthesised as previously described (Murdoch *et al.,* 2004). CORM-308 was dissolved in DMSO and used freshly on the day of experiment. When assessed by myoglobin assay, CORM-308 was found to release CO rapidly ($t_{1/2}$ ~5 min) and approximately one mol of CO is released by one mol of CORM-308 (unpublished data from our group). The inactive form (iCORM-308), which does not release CO and was used as negative control, was prepared in DMSO solution and left at least for 48 h to decompose. The iCORM-308 solution was finally bubbled with a stream of nitrogen to remove any residual CO present in the solution (unpublished data from our group).

2.1.6 *Synthesis of CORM-314 ($[Fe(CO)_3Cl(\eta^3\text{-}C_3H_5)]$)*

CORM-314 was synthesised as previously described (Murdoch *et al.,* 2004). CORM-314 was dissolved in DMSO and used freshly on the day of experiment. When assessed by myoglobin assay, CORM-314 was found to release CO very rapidly ($t_{1/2}$ ~1 min) and approximately one mol of CO is released by one mol of CORM-314 (unpublished data from our group). The inactive form (iCORM-314), which does not release CO and was used as negative control, was prepared in DMSO solution and left at least 48 h to decompose. The iCORM-314 solution was finally bubbled with a stream of nitrogen to remove any residual CO present in the solution (unpublished data from our group).

2.2 Preparation of Aortic Rings

Aortic rings preparation was carried out as previously described (Scott *et al.,* 1996; Foresti *et al.,* 2004). Male Sprague-Dawley rats (300-400g) were killed by cervical dislocation and exsanguination. The thoracic aortic artery was extracted and immediately stored in cold Krebs Henseleit solution (4°C). After gentle flushing with cold Krebs Henseleit solution, the aorta from each animal was cleared of surrounding connective tissue and cut into four rings ~2 mm in length. Then the aortic rings were carefully mounted between two stainless steel wires in 18-ml organ baths. The organic baths contained Krebs Henseleit buffer (composition in mM) : (NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 22, glucose 11, K^+ EDTA 0.03, $CaCl_2$ 2.5) and the rings were left to equilibrate for 1 h before any experiments were conducted. Then the rings were contracted with a standard dose of KCL (110 mM) in order to provide an internal reference of contraction. Indomethacin (10 μM) was added to the Krebs Henseleit buffer to rule out prostaglandins as potential modulators of vascular tone. The buffer was aerated with 95% O_2-5% CO_2 and maintained at 37° C. The isolated blood vessels were maintained under a resting tension of 2 g. One wire was fixed to a metal handle and the other was attached to an isometric force transducer and a Grass model 7D polygraph for continuous recording of tension (**Figure 2.1**).

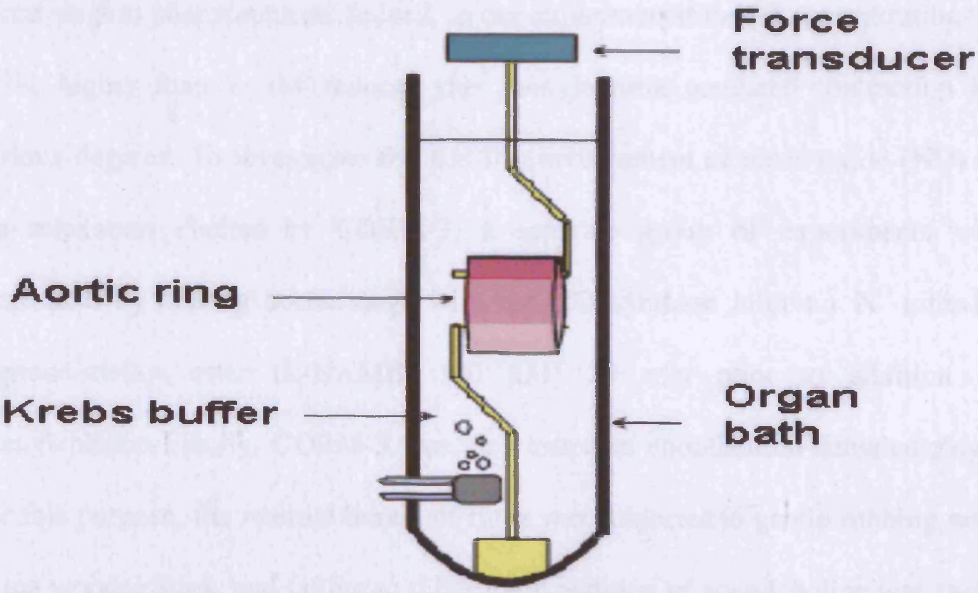


Figure 2.1. Isolated aortic ring model (from Dr Motterlini)

2.2.1 Aortic ring preparation group (1): vasoactive properties of CORM-3

To establish the potential vasorelaxant effects of CORM-3, aortic rings were precontracted with phenylephrine (1 μM) before addition of CORM-3 at different concentrations (25–200 μM). CORM-3 was added three times at 6–10 min intervals. In another experiment the response of precontracted aortic rings to one addition of CORM-3 (100 μM) was monitored over 1 h. Because CO gas has been reported to activate guanylate cyclase to increase the production of cGMP (Hussain *et al.,* 1997; Nakatsu *et al.,* 2002), additional experiments were conducted to examine the involvement of cGMP in the relaxation mediated by CORM-3. For this, aortic rings were incubated with the inhibitor of guanylate cyclase ODQ (10 μM) 15 min prior to addition of phenylephrine. The effect of CORM-3 on vascular tone was also assessed in the presence of the stimulator of soluble guanylate cyclase, YC-1 (1 μM), which was incubated 30 min prior to addition of phenylephrine. Preliminary experiments were conducted in order to identify the concentration of YC-1 that did not significantly affect the response of aortic rings to phenylephrine. Indeed, in our experimental model concentrations of YC-1 higher than 1 μM reduced the phenylephrine-mediated contraction by various degrees. To investigate the possible involvement of nitric oxide (NO) in the relaxation elicited by CORM-3, a separate group of experiments was performed by treating aortic rings with the NO synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) 30 min prior to addition of phenylephrine. Finally, CORM-3 was also tested in endothelium-denuded rings. For this purpose, the internal lumen of rings was subjected to gentle rubbing with a fine wooden stick, and failure to dilate upon addition of acetylcholine was taken as a proof of successful endothelium removal.

2.2.2 Aortic ring preparation group (2): vasoactive properties of CORM-A1

To establish the potential vasorelaxant effects of CORM-A1, aortic rings were precontracted with phenylephrine (1 μM) before addition of the compound at different concentrations (40-160 μM). The extent of vasorelaxation over one hour was determined and compared with the effect produced by one addition of CORM-3 (100 μM). In another set of experiments, the inactive form (iCORM-A1) and sodium borohydride ($NaBH_4$) were used as negative control to exclude the possibility that vasorelaxation was a non-specific effect of boron compounds or borate. In addition myoglobin (80 μM) was added simultaneously with CORM-A1 (80 μM) to verify the role of CO in mediating the vasoactivity of CORM-A1. Additional experiments were conducted to examine the involvement of cGMP in relaxation mediated by CORM-A1. For this purpose, ODQ (30 μM), an inhibitor of guanylate cyclase, was added simultaneously with CORM-A1 to contracted aortic rings. The effect of CORM-A1 on vascular tone was also assessed in the presence of a stimulator of soluble guanylate cyclase, YC-1 (1 μM), which was added 30 min before phenylephrine. To investigate the possible involvement of NO in the relaxation elicited by CORM-A1, a separate group of experiment was performed by treating aortic rings with the NO synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) 30 min before addition of phenylephrine. CORM-A1 was also tested in endothelium-denuded rings. For this, the internal lumen of rings was subjected to gentle rubbing with a fine wooden stick and failure to dilate upon addition of acetylcholine was taken as a proof of successful endothelium removal.

2.2.3 *Aortic ring preparation group (3): assessment of the role of K^+ channels in mediating the vasorelaxing properties of CORM-3 and CORM-A1.*

Potassium (K^+)channels, which comprise the most sophisticated ion channel superfamily in mammalian cells, play an important role in regulating vascular tone and mediating the action of a large number of vasoactive agents (Nelson and Quayle, 1995; Cao *et al.,* 2002). K^+ channel inhibitors, which are invaluable for investigating the properties and functional role of K^+ channels (Olesen *et al.,* 1994; Nelson and Quayle, 1995), were used to assess the involvement of different K^+ channels subgroups in mediating the vasorelaxing properties of CORM-3 and CORM-A1. For this purpose transverse sections of aortic rings were prepared as described above and divided into six groups. Each group was treated with different K^+ channel subgroup inhibitors for 30-60 min before contracting the rings with phenylephrine (1-1.5 μM) then the response of contracted rings to a single addition of CORM-A1 (80 μM) or three consecutive additions of CORM-3 (100 μM) was measured over time as mentioned above. The first group was incubated for 60 min with 30 mM of tetraethyammonium (TEA), a nonslective K^+ channel inhibitor which blocks K_{Ca}, K_{ATP}, and K_V channels. The second group of rings were pretreated with glibenclamide (GLI 10 μM), the K_{ATP} channel inhibitor for 30 min, to assess the involvement of K_{ATP} channels. The third group was incubated with 1 mM of 4-aminopyridine (4-AP), a selective K_V channel blocker, for 30 min. The fourth group was incubated for 60 min with charybdotoxin (100 nM), the selective blocker of big conductance Ca^{+2} activated K^+ channels (BK_{Ca} channels), whereas the fifth group was incubated with apamine (100 nM), the selective inhibitor of small conductance Ca activated K^+ channels (SK_{Ca} channels). Finally, to confirm the possible involvement of both K_{Ca} channel subtypes in amplifying the signalling effect of CORM-3 and CORM-A1, a sixth group of aortic rings was incubated with both apamine (100 nM) and charybdotoxin (100 nM) simultaneously for 60 min before contracting the rings with phenylephrine.

Knowing that KCl at high concentration might selectively block K^+ channels, in a separate set of experiments we tested the ability of CORM-A1 and CORM-3 to induce vasorelaxation in high KCl contracted rings. For this purpose, groups of aortic rings were maximally contracted with KCl (110 mM) solution, and then the response of these rings to a single addition of CORM-A1 (80 μM) or three additions of CORM-3 (100 μM) was measured over time.

2.2.4 Aortic ring preparation group (4): vasoactive properties of iron-containing CO-RMs

The aortic rings were prepared as described above and the extent of vasorelaxation by a single addition of CORM319 (12.5-100μM) over one hour was monitored in aortic rings precontracted with phenylephrine (1 μM) and compared to the effect induced by iCORM-319 (100 μM), the inactive form which does not release CO. Similarly, the vasorelaxing properties of single addition of DMSO soluble CORM-307, 308 and 314 (100 μM) were also assessed and compared to their inactive forms (iCO-RMs). Vasodilatory response was expressed as a percentage of the maximum vasoconstriction induced by phenylephrine.

2.3 Cell Culture

The rat aortic smooth muscle cell line A7r5 was obtained from the European Collection of Cell Culture (ECCAC-UK) and was used at passages 12-20 following supplier's instruction (Lizard *et al.,* 1999; Zahm *et al.,* 2003). Cell were grown and passaged in 75 mm^2 plastic culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.4, in a humidified incubator at 37 C° with 5 % CO_2 atmosphere. At 60-80 % confluence, which was usually reached after 5-6 days, the culture medium was decanted and the monolayer of cells was washed with 10 ml PBS and the cells were released from culture flasks using 4 ml Trypsin/EDTA solution. Then the cells were resuspended in warm medium solution and centrifuged for 5 min. After that the harvested cells were seeded in 75 ml flasks with a split ratio of 1:3.

2.4 Alamar Blue Assay

The harvested A7r5 cells from each flask were suspended in medium solution and seeded into two 24 well plates (each well contained 1 ml medium solution) for 24 h. Next day the medium was removed and a new 1 ml of medium containing the required concentration of CO-RM under study was added to each well and left in the incubator for 24 h. The following day Alamar Blue solution was prepared freshly by dissolving 1 part of Alamar Blue to 9 parts of medium solution to have 10 % Alamar Blue solution (Nakayama *et al.,* 1997; O'Brien *et al.,* 2000). Then the medium containing CO-RM treatment was removed from the plates and 0.5 ml of 10 % Alamar Blue solution was added to each well. The plates were left in the incubator for 3 h; during this period the blue colour of the medium gradually changed to pink. After that 200 μM from each well were transferred into 96 wells plate in order to be read by microplate reader. Alamar Blue assay is based on the detection of metabolic activity, particularly of the metabolic chain reaction, of living cells utilizing a redox indicator which changes from oxidized (dark blue) form to reduced (red) form (O'Brien *et al.*, 2000; Sawle *et al.,* 2005). The extent of this conversion which is proportional to cell metabolism, is calculated as the difference in absorbance between 570 and 600 mm and expressed as percentage of control (Sawle *et al.*, 2005).

2.4.1 Alamar Blue assay group (1): effect of CORM-3 on aortic SMCs viability

Rat aortic smooth muscle cells (A7r5) were cultured as described above. Monolayers of A7r5 cells in 24 wells plates were incubated with CORM-3 (25-1000 μM) and iCORM-3 (100-1000 μM) solutions for 24 h. Then the cell viability was determined in aortic smooth muscle cells using an Alamar Blue viability assay kit as described above.

2.4.2 Alamar Blue group (2): effect of CORM-A1 on aortic SMCs viability

Rat aortic smooth muscle cells (A7r5) were cultured as described earlier. Monolayers of A7r5 cells in 24 well plates were incubated with CORM-A1 (10-1000 μM) and iCORM-A1 (100-1000 μM) solutions for 24 h. Then the cell viability was determined in aortic smooth muscle cells using an Alamar Blue viability assay kit as described above.

2.4.3 Alamar Blue assay group (3): effect of iron-containing CO-RMs on aortic SMCs viability.

Rat aortic smooth muscle cells (A7r5) were cultured as previously described. Monolayers of A7r5 cells in 24 well plates were divided into four groups. The first group was incubated with CORM-319 (10-500μM) and its inactive form iCORM-319 (10-500 μM) for 24 h. The other three groups were treated for 24 h with increasing concentrations (10-100μM) of active and inactive forms of CORM-307, 308 and 314. Then the cell viability was determined using an Alamar Blue viability assay kit as described above.

2.5 Determination of cGMP Levels

Tissue sample purification, reagent preparation and assay method were conducted following manufacture's instructions.

2.5.1 Tissue sample collection

Male Sprague-Dawley rats (300-400g) were killed by cervical dislocation and exsanguination. The thoracic aorta was removed and flushed with Krebs-Henseleit buffer and immediately stored in cold Krebs-Henseleit buffer. The aorta from each animal was cleared from surrounding tissue and allowed to recover for 1 h in oxygenated Krebs–Henseleit buffer before treatment with phenylephrine (1 μM). Aortas were immediately immersed in liquid nitrogen at -196°C for 8 min following one, two or three consecutive bolus additions of CORM-3 (100 μM), each addition given at 8 min intervals. This interval was chosen based on the time required by aortic rings to reach a stable relaxation upon addition of CORM-3. The aortas were stored at -70°C immediately after collection to prevent alteration to the cGMP and associated enzymes before analysis.

2.5.2 Tissue sample purification

The frozen tissue was homogenised in cold 6% (w/v) trichloroacetic acid at 2-8^{0}C to give a 10% (w/v) homogenate. The homogenate was centrifuged at 2000xg for 15 minutes at 4^{0}C, then the supernatant was recovered and the pellet was discarded. The supernatant was washed four times with five volumes of water saturated diethyl ether. The upper ether layer was discarded after each wash. The aqueous extract remaining was dried under a stream of nitrogen at 60°C then it was stored at -70^{0}C.

2.5.3 Reagent preparation

Five reagents were prepared for this assay. Assay buffer (500 ml) and wash buffer

(500 ml) were prepared in distilled water. Standard reagent (92.5ml), antibody reagent (11ml) and cGMP conjugate (11ml) were prepared in assay buffer. These reagents were used to prepare nine working standard Eppendorf tubes (labelled 2, 4, 8, 16, 32, 64, 128, 256 and 512) according to manufacture instruction (Amersham Biosciences) freshly on the day of assay.

2.5.4 Assay method

Assay buffer and working standards were prepared as described above. Eppendorf tubes were labelled for zero, standards and unknowns. These tubes were known as acetylation tubes. A 96 well microplate was prepared for running of all blanks, standards and samples in duplicate. Acetylation reagent was prepared by adding 1 volume acetic anhydride to 2 volumes of triethylamine. One ml of diluted assay buffer was pipetted into zero standard acetylation tube. One ml of each unknown, which were prepared earlier, was pipetted into properly labelled acetylation tubes. 100 μM of acetylation reagent was added to all acetylation tubes containing standards and unknowns. 100 μM of antiserum was pipetted into all wells except the blank and non-specific binding (NSB) wells. Then starting with the most dilute, duplicates of 50 μl aliquots from all acetylation tubes were pipetted into appropriate wells. This step was followed by pipetting 150 μl of assay buffer into the non-specific binding wells. Then the plate was incubated at 3-5 C^{o} for two hours. Two hours later all wells, except the blank, were pipetted with 100 μl diluted conjugate and incubated at 3-5 C^{o} for one hour. Then all wells, after aspiration and thorough washing, were pipetted with 200 μl of equilibrated enzyme substrate and mixed on microplate shaker for 30 min at room temperature. Finally the blue colour that developed was read at 630 nm in microplate reader.

2.6 Chemicals

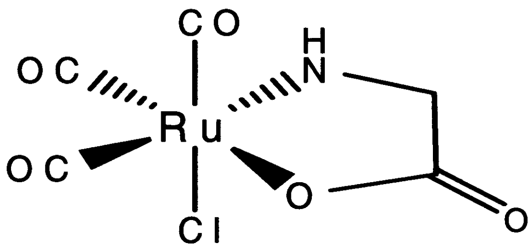
1*H*-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ), glibenclamide and 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) were from Alexis Corporation (Nottingham, U.K.). Alamar Blue viability assay kits were purchased from Serotec Inc, Kidlington, Oxford, UK. CORM-3 was synthesised and supplied by Professor Brian Mann and co-workers, Department of Chemistry, University of Sheffield, Sheffield, UK. CORM-A1 was supplied by Dr Roger Alberto, Department of Chemistry, University of Zurich, Switzerland. All other chemicals used in the study were from Sigma, unless otherwise specified.

2.7 Statistics

Vasodilatory response was expressed as a percentage of the vasoconstriction induced by phenylephrine. Statistical analysis was performed using two-away Anova combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$. Statistical analysis of the cell viability was performing using one-way Anova combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$.

3 VASOACTIVE PROPERTIES OF CORM-3

Tricarbonylchloro(glycinato)ruthenium(II), which has been termed CORM-3, is a pharmacologically active water-soluble metal carbonyl complex, which was obtained by coordinating glycine onto the metal center as shown below (Motterlini *et al.,* 2003; Foresti *et al.,* 2004).



Tricarbonylchloro(glycinato)ruthenium(II)
CORM-3

Graph 3.1A. Chemical structure of CORM-3 (adapted from Motterlini *et al.,* 2003)

CORM-3 releases CO *in vitro, ex vivo* and *in vivo* biological models (Motterlini *et al.,* 2003) and has been reported to have protective effects against ischaemia-perfusion injury in rats and cardiac allograft rejection in mice (Clark *et al.,* 2003). Further more, CORM-3 has been shown to attenuate inflammatory response elicited by lipopolysaccharide in murine macrophages (Sawle *et al.,* 2005a). These results prompted us to further investigate the vasoactive properties of CORM-3. For this purpose we investigated the vasorelaxing effect of CORM-3 on an *ex vivo* model of isolated aortic rings. In addition we tried to explore the possible mechanisms that might mediate the CORM-3-induced vasorelaxation on aortic rings. Among different proposed pathways we focused on soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway as a possible target for CORM-3. The role of endothelium and nitric oxide gas (NO) in mediating the vasorelaxing activities of CORM-3 was also investigated in this project. Finally we did not forget to assess the effect of CORM-3 on aortic smooth muscle cells (SMCs) viability *in vitro* to rule out any toxic effects for CORM-3 on vascular SMCs.

3.1 Material and Methods

3.1.1 Preparation of CORM-3 solution

CORM-3 was synthesized as previously described (Clark *et al.,* 2003; Motterlini *et al.,* 2003). CORM-3 was freshly prepared before the experiments by dissolving the compound in distilled water. Inactive CORM-3 (iCORM-3) was obtained by leaving CORM-3 in Krebs-Henseleit buffer overnight at room temperature. This treatment produced a compound that does not release CO and therefore, iCORM-3 was used as a negative control to assess the direct involvement of CO in the pharmacological actions of CORM-3 (Clark *et al.,* 2003; Foresti *et al.,* 2004). It was reported that around 1 mol of CO per 1 mol of CORM-3 is liberated within 10 min after addition to Krebs-Henseleit buffer (Clark *et al.,* 2003; Foresti *et al.,* 2004).

3.1.2 Aortic ring preparations

Transverse sections of aortic rings were prepared from adult Sprague-Dawley rats (300-400 g) and suspended under 2 g tension in oxygenated Krebs-Henseleit buffer as previously described in Materials and Methods (Chapter 2). To establish the potential vasorelaxant effects of CORM-3, aortic rings were precontracted with phenylephrine (1 μM) before addition of CORM-3 at different concentrations (25–200 μM). CORM-3 was added three times at 6–10 min intervals. In another experiment the response of precontracted aortic rings to one addition of CORM-3 (100 μM) was monitored over 1 h. Additional experiments were conducted to examine the involvement of cGMP in the relaxation mediated by CORM-3. For this purpose, aortic rings were incubated with the inhibitor of guanylate cyclase ODQ (10 μM) 15 min prior to addition of phenylephrine. The effect of CORM-3 on vascular tone was also assessed in the presence of the stimulator of soluble guanylate cyclase, YC-1 (1 μM), which was incubated 30 min prior to addition of phenylephrine. Preliminary experiments were conducted in order to identify the concentration of YC-1 that did not significantly affect the response of aortic rings to phenylephrine. Indeed, in our experimental model concentrations of YC-1 higher than 1 μM reduced the phenylephrine-mediated contraction by various degrees. To investigate the possible involvement of nitric oxide (NO) in the relaxation elicited by CORM-3, a separate group of experiments was performed by treating aortic rings with the NO synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) 30 min prior to addition of phenylephrine. Finally, CORM-3 was also tested in endothelium-denuded rings. For this purpose, the internal lumen of rings was subjected to gentle rubbing with a fine wooden stick, and failure to dilate upon addition of acetylcholine was taken as a proof of successful endothelium removal.

3.1.3 Determination of aortic cGMP levels

Aortas were collected and allowed to recover for 1 h in oxygenated Krebs–Henseleit buffer before treatment with CORM-3. Aortas were freezed-clamped 8 min following one, two or three consecutive bolus additions of CORM-3 (100 μM), each addition given at 8 min intervals. This interval was chosen based on the time required by aortic rings to reach a stable relaxation upon addition of CORM-3. Levels of cGMP were measured in aortic tissue extracts using a commercial ELISA kit (Amersham) following the manufacturers' instructions. Four aortas per group were used for the assay. cGMP levels were expressed as fmol mg^{-1} of aortic tissue.

3.1.4 Cell viability assay

Rat aortic smooth muscle cells (A7r5) were cultured as previously described in Materials and Methods (Chapter 2). Monolayers of A7r5 cells in 24 wells plates were incubated with CORM-3 (25-1000 μM) and iCORM-3 (100-1000 μM) solutions for 24 h. Then the cell viability was determined in aortic smooth muscle cells using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK) as previously explained in Material and Methods.

3.1.5 Statistical analysis

Vasodilatory response was expressed as percentage of the vasoconstriction induced by phenylephrine. Statistical analysis was performed using one-way and two-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P<0.05$.

3.2 Results

3.2.1 Effect of CORM-3 on vascular tone in vitro

Figure 3.1 shows that CORM3 induced a concentration-dependent vasorelaxation on precontracted aortic rings. The vasorelaxing response to first addition of CORM-3 (100 μM) was 36.5 % increased to 47.8 % and 52.7 % after second and third additions respectively. The relaxation induced by 200 μM CORM-3 did not differ significantly from that induced by 100 μM (44.4 vs. 36.5 % after first addition respectively). In contrast, iCORM-3 (100 μM) did not elicit evident relaxation, demonstrating that CO released from CORM-3 is the factor involved in the modulation of vessel tone. The vasorelaxing response to one addition of CORM-3 (100 μM) reached the maximum within 10 min and remained sustained without recontraction over one hour **(Figure 3.2)**.

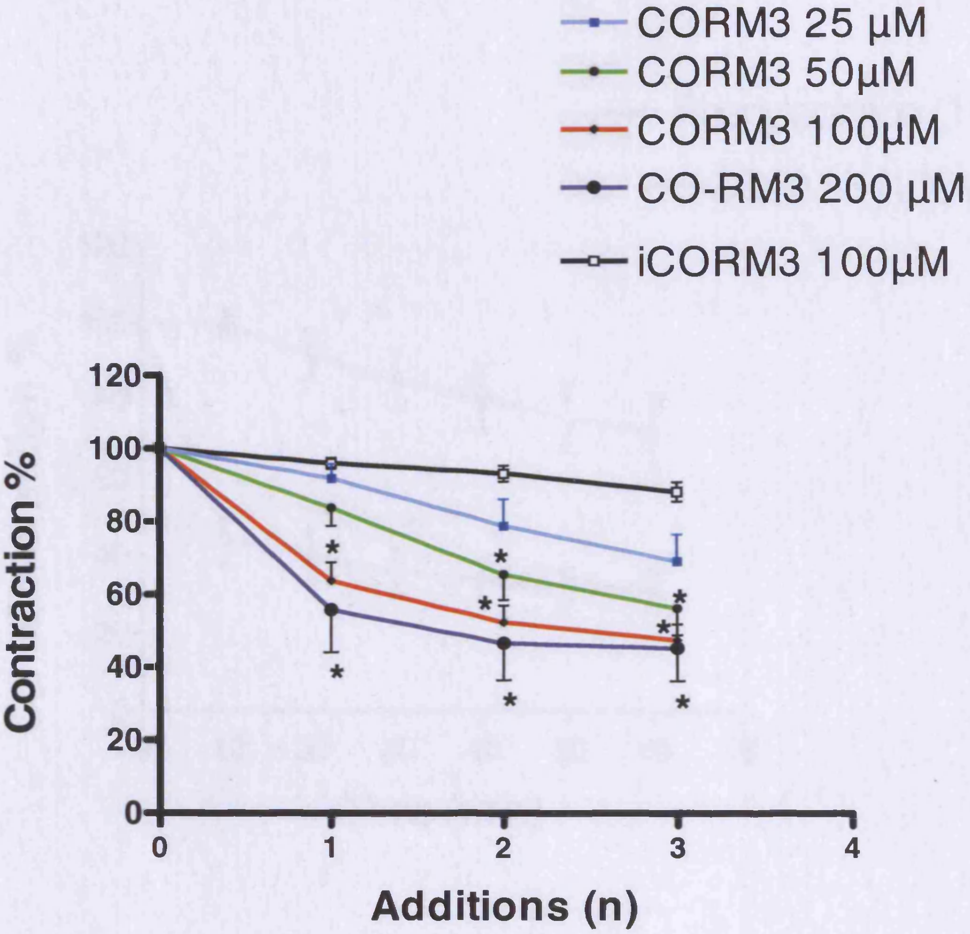


Figure 3-1. Effect of CORM-3 on vascular tone.

This graph shows the vasodilatory response of aortic rings subjected to three consecutive bolus additions of CORM-3 at different concentrations (25-200 μM). iCORM-3 (100 μM), the negative control, did not cause any evident relaxation. Vasodilation is expressed as the percentage of maximal precontraction. Data represent the mean ±s.e.m. of 6-8 independent experiments. *P< 0.05 compared to iCORM-3.

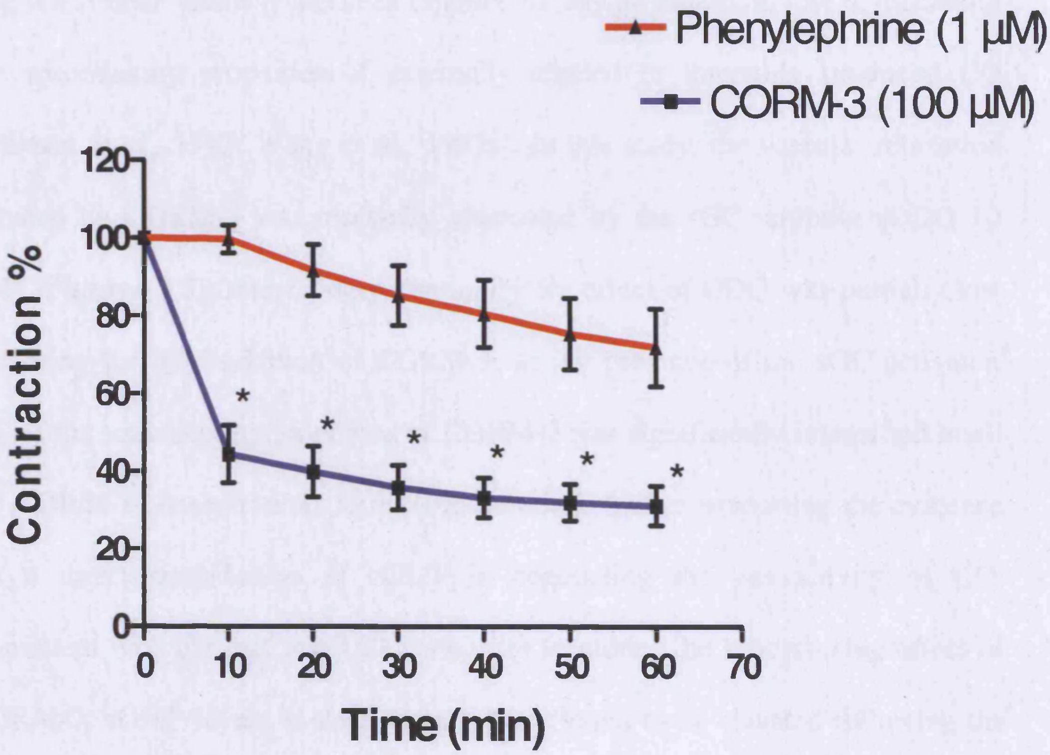


Figure 3.2. Effect of CORM-3 on vascular tone.

The response of aortic rings precontracted with phenylephrine (1 µM) to one addition of 100 µM CORM-3 was monitored over one hour. CORM-3 induced significant and sustained vasorelaxation on precontracted rings. Vasodilation is expressed as the percentage of maximal precontraction. Data represent the mean ±s.e.m. of 5-6 independent experiments. *$P < 0.05$ compared to phenylephrine.

3.2.2 Involvement of the sGC/cGMP pathway in mediating the vasorelaxing effect of CORM-3

The sGC/cGMP pathway has been reported to play an important role in mediating the vasorelaxing properties of externally applied or internally produced CO (Hussain *et al.,* 1997; Wang *et al.,* 1997a). In this study, the vascular relaxation induced by CORM-3 was markedly attenuated by the sGC inhibitor (ODQ 10 μM) **(Figures 3.3)**. Interestingly, the inhibitory effect of ODQ was partially lost following the third addition of CORM-3. In the presence of the sGC activator, YC-1, the vasorelaxing properties of CORM-3 was significantly intensified at all the CORM-3 concentrations tested **(Figures 3.4)**, further supporting the evidence for a direct contribution of cGMP in conducting the vasoactivity of CO. Consistent with the fact that ODQ was able to reduce the vasorelaxing effect of CORM-3, cGMP levels in aortic tissues were found to be elevated following the first and second addition of CORM-3 (100 μM) **(Figure 3.5)**. cGMP was significantly augmented from a basal level of 0.26±0.03 (control group, CON) to 1.23±0.3 and 0.97±0.4 fmol mg^{-1} tissue ($P<0.05$) after the first and second addition of CORM-3, respectively. In contrast, addition of a third bolus of CORM-3 did not change cGMP levels compared to control (0.27±0.02 fmol mg^{-1} tissue).

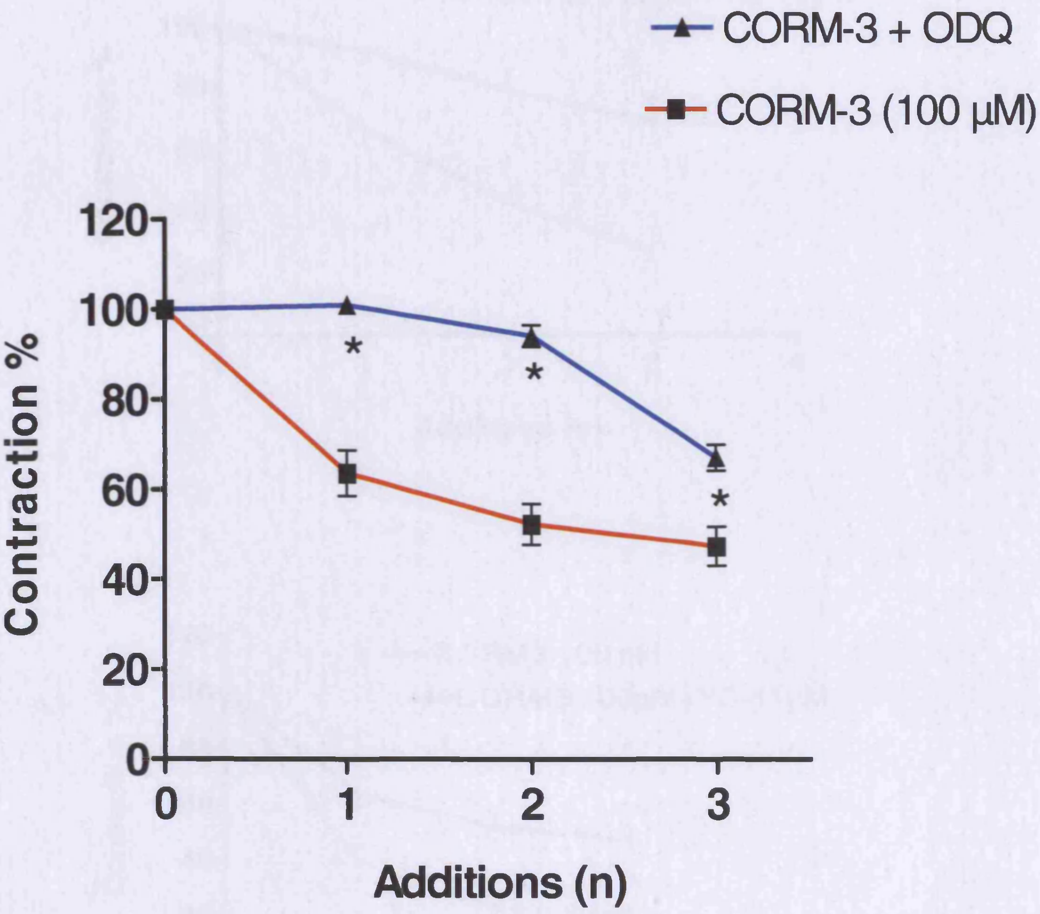
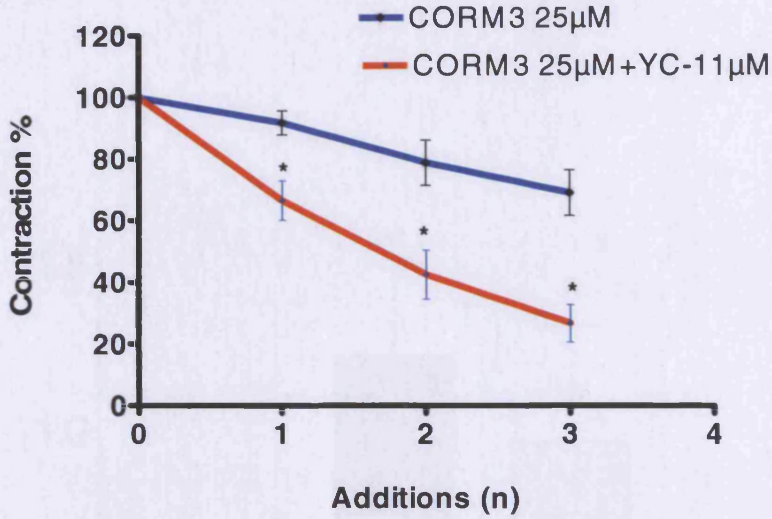


Figure 3-3. Vasorelaxing effect of CORM-3 in the presence of ODQ.

This graph shows the vasodilatory response of aortic rings subjected to three consecutive bolus additions of CORM-3 in the presence of ODQ (10 µM). ODQ was added to the water bath 15 min prior to the addition of phenylephrine. ODQ significantly attenuated the vasorelaxing effect of CORM-3. Dilatation is expressed as percentage of precontraction. Data represent the mean±s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to 100 µM CORM-3 alone.



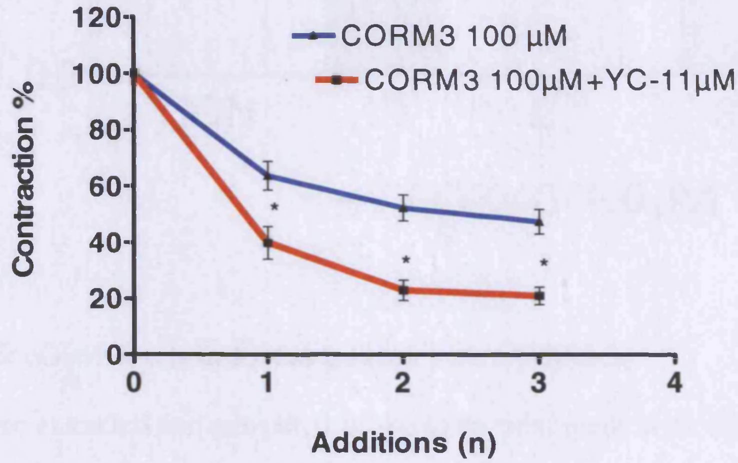


Figure 3.4. YC-1 potentiates CORM-3-induced vasorelaxation.

This graph shows the vasodilatory response of aortic rings subjected to three consecutive bolus additions of CORM-3 (25 and 100 µM) in the presence of 1 µM YC-1. As shown (a and b) the presence of YC-1 potentiated the vasodilatory effect of CORM-3 at both concentrations. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-3 alone.

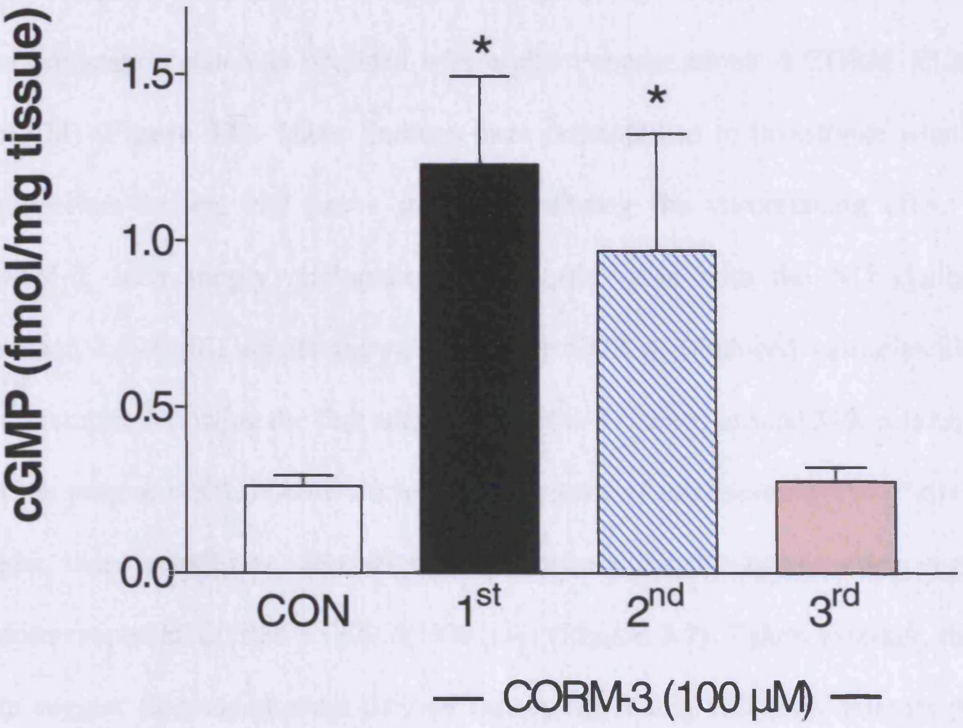


Figure 3.5. cGMP levels in aortas treated with CORM-3.

Aortas were extracted and subjected to the same treatments with CORM-3 as for measurement of isometric tension. At 8 min after the first, second or third addition of CORM-3 (100 μM), aortas were frozen and subsequently processed for cGMP assay. Control aortas (CON) were treated with vehicle (water). Data represent the mean ± s.e.m. of four different aortas per group. $^{*}P<0.05$ compared to control.

3.2.3 Effect of CORM-3 on vascular tone of aortic rings pretreated with a NO synthase inhibitor or endothelium-denuded rings

To assess the impact of intact endothelium on CORM-3 vasoactivity, CORM-3 was tested in endothelium-denuded rings. Intriguingly, removal of the endothelial layer completely abolished the relaxation elicited by 100 μM CORM-3, and a small relaxation was only obtained with higher concentrations of CORM-3 (200-400 μM) **(Figure 3.6)**. These findings have prompted us to investigate whether endothelium-derived NO has a role in mediating the vasorelaxing effect of CORM-3. Interestingly, preincubation of aortic rings with the NO synthase inhibitor, L-NAME, significantly reduced the CORM-3-induced vasorelaxation. For example, following the first addition, CORM-3 elicited around 37% relaxation but the presence of L-NAME decreased the extent of relaxation to 5% (P<0.05). Under these conditions, relaxation could be elicited by using much higher concentrations of CORM-3 (200 or 400 μM) **(Figure 3.7).** Taken together, these data suggest that endothelium-derived factors, including NO, may interact with CORM-3 and potentiate its pharmacological activities.

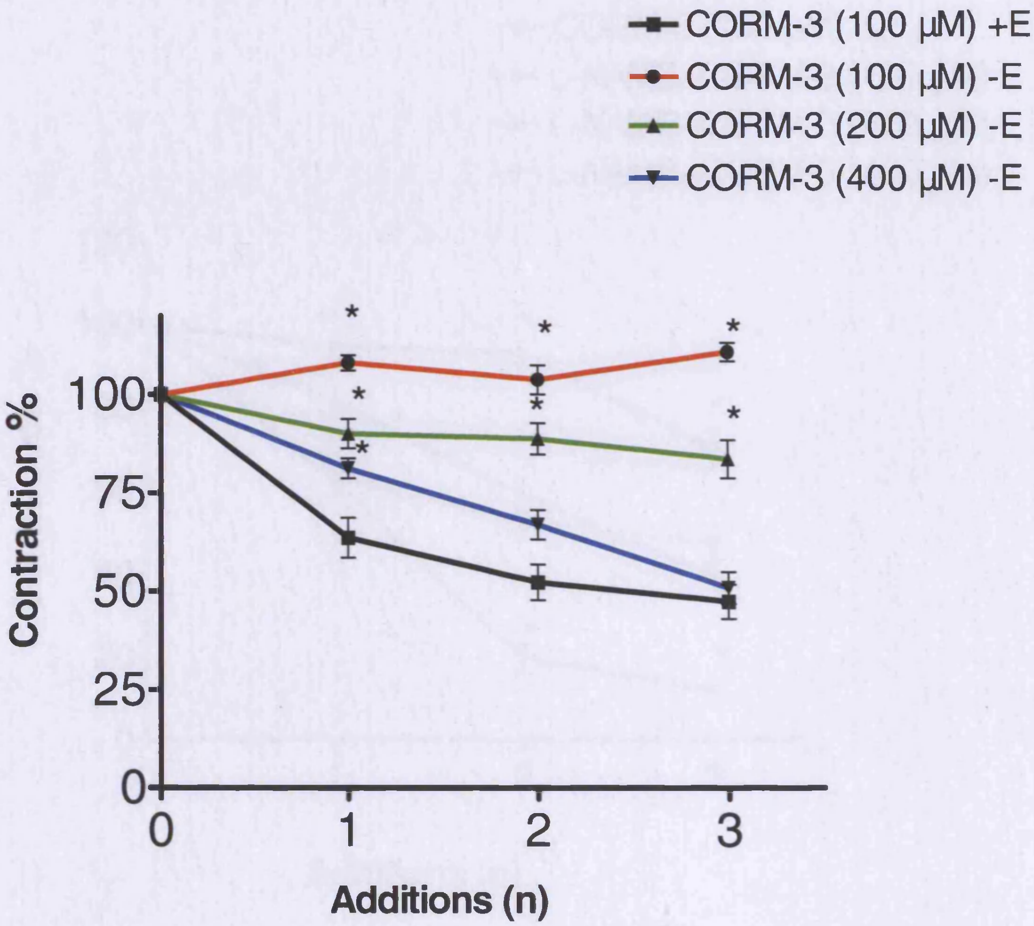


Figure 3.6. Removal of the endothelium reduces CORM-3-mediated dilatory response.

The endothelium of aortic rings was gently removed (E^-) as described in Methods and the response to three consecutive bolus additions of CORM-3 at different concentrations (100, 200 and 400 μM) was compared to that of intact rings E^+. The absence of the endothelium markedly reduced CORM-3-mediated vasodilation and higher CORM-3 concentration were required to produce relaxation. Data represent the mean ± s.e.m. of 6-8 independent experiments. $^*P < 0.05$ compared to 100 μM CORM-3 in intact endothelium.

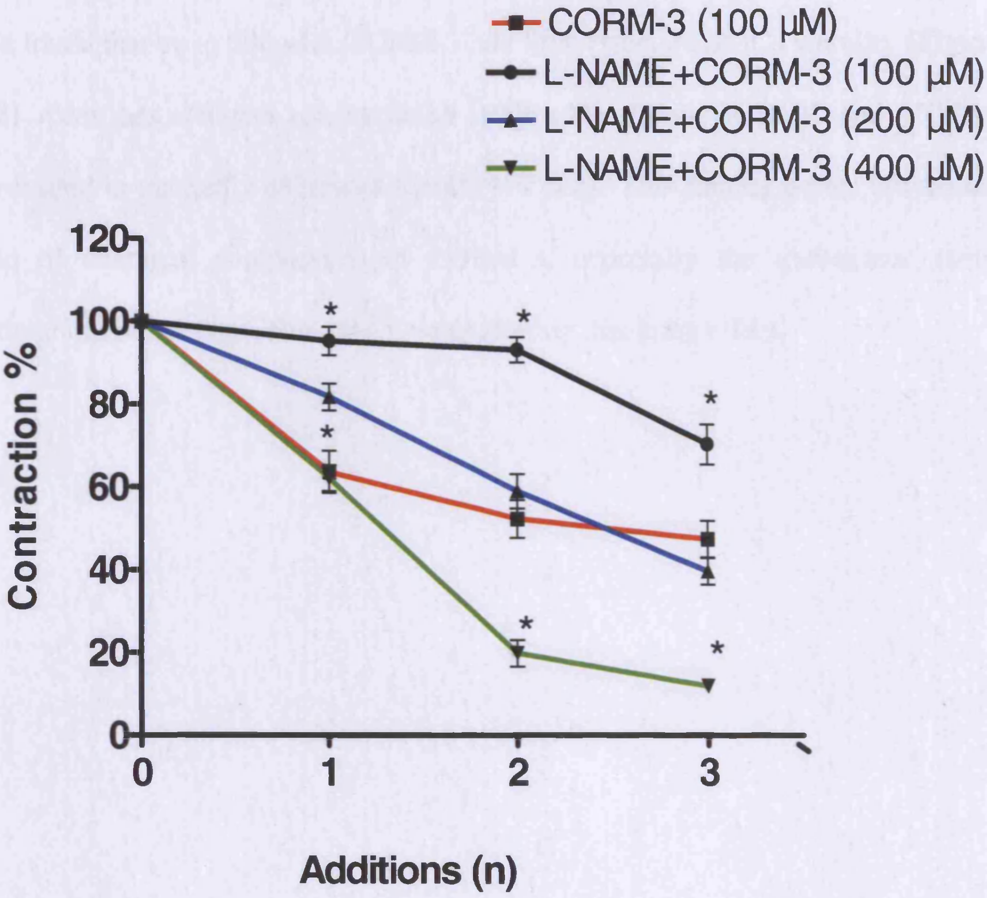


Figure 3.7. Blockade of NO production reduces CORM-3-mediated dilatory response.

Aortic rings were incubated with the inhibitor of the NO synthase pathway L-NAME (100 μM) for 30 min prior to contraction with phenylephrine. L-NAME markedly prevented relaxation caused by 100 μM CORM-3, and higher concentration of CORM-3 (200-400 μM) were required to induce dilatation in the presence of L-NAME. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P <0.05 compared to 100 μM CORM-3 alone.

3.2.4 Effect of CORM-3 on SMCs viability

Because CORM-3 contains ruthenium which is a heavy metal and has a potential toxic effect, we assessed the effect of CORM-3 on cultured aortic SMCs *in vitro.* We found that up to 500 μM, CORM-3 had little effect on SMCs viability **(Figure 3.8)**. Additions at higher concentration (1000 μM) of both CORM-3 and iCORM-3 resulted in markedly depressed viability of cells. This finding points toward the role of chemical components of CORM-3, especially the transitional metal ruthenium, rather than released CO in mediating this toxic effect.

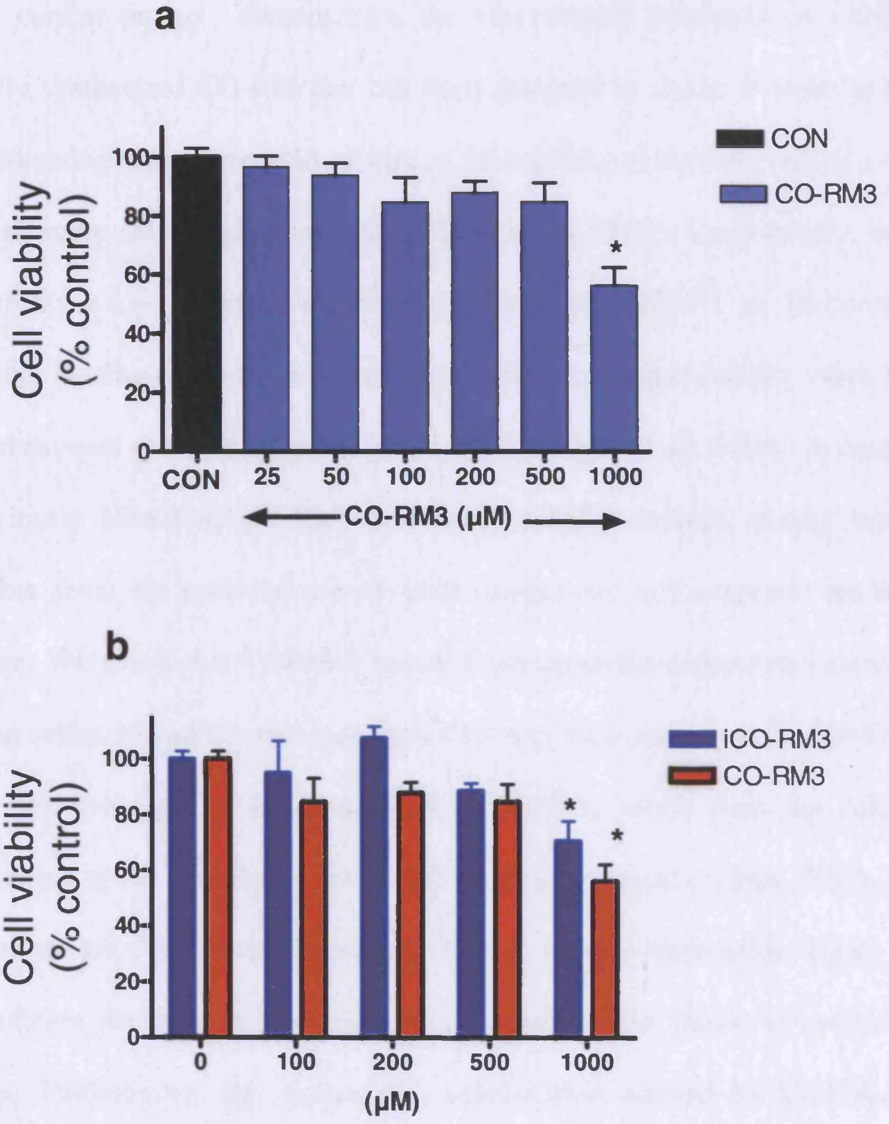


Figure 3.8. Effect of CORM-3 on cell viability.

Aortic SMCs (A7r5) were treated with increasing concentrations of CORM-3 (25-1000 μM) and iCORM-3 (100-1000 μM) for 24 h and the change in cell viability was assessed using Alamar Blue technique. As shown in figure (a) CORM-3 up to 500 μM had no significant toxic effect on SMCs. At higher concentration (1000 μM) CORM-3 induced considerable loss of cell viability. Figure (b) shows that the inactive form iCORM-3 was slightly less toxic than CORM-3. Data represent the mean ± s.e.m. of 6-12 independent experiments. *P<0.05 compared to control.

3.3 Discussion

The current project demonstrates the vasorelaxant properties of CORM-3, a newly synthesized CO-RM that has been designed to render it water soluble by coordinating the amino acid glycine, a biologically compatible ligand , onto the metal centre (Motterlini *et al.,* 2003; Clark *et al.,* 2003). Undoubtedly, the water solubility is a significant evolution in the design of CORM-3 as the more water-soluble the drugs are, the better their absorption and bioavailability when they are administrated orally (McFarland *et al.,* 2003; Berger *et al.,* 2004). In contrast the previously identified CO-RMs were only DMSO soluble, posing reasonable doubts about the potential use of these compounds as therapeutic medicines in future. We found that CORM-3 induced concentration-dependant vasorelaxation of precontracted aortic rings and this effect was clear starting at 25 μM CORM-3. The inactive form of the compound, iCORM-3, which does not release CO according to the myoglobin assay, did not induce vasorelaxation. These findings point toward CO liberated from CORM-3 as the responsible factor for the significant decrease in vascular tone observed in our model of isolated aortic rings. Furthermore, the pattern of vasorelaxation elicited by CORM-3 might reflect its kinetics of CO liberation according to the myoglobin assay. The finding that the half life of CO is around 5 min might explain the significant decrease in vascular tone that happened within 10 min from administration of CORM-3 into organic baths.

The presence of ODQ, a sGC inhibitor, partially attenuated the vasorelaxation of aortic rings induced by CORM-3. This finding indicates that sGC/cGMP is a possible cellular mediator of CORM-3-induced vasorelaxation. The role of sGC/cGMP pathway was further supported by elevated cGMP levels measured in aortic tissues following the addition of CORM- 3. Our data accord with previous studies in which it has been proposed that the sGC/cGMP cellular pathway is the cellular target of endogenously produced or externally applied CO (Hussain *et al.*, 1997; Wang *et al.* 1997a; Nakatsu *et al.*, 2002). In addition, the finding that YC-1, which has been reported to promote the stimulatory effect of CO on pure sGC, remarkably potentiated the vasorelaxing properties of CORM-3, strongly supports the role played by sGC/cGMP pathway. In the presence of YC-1 lower concentrations of CORM-3 were needed to elicit significant vasorelaxation. Similar findings have been observed when external CO was combined with YC-1(McLaughlin *et al.*, 2000). Therefore, the combination of CORM-3 and YC-1 might have important therapeutic application in vascular diseases.

A remarkable finding of the current study is that the vasorelaxation induced by CORM-3 was significantly reduced in endothelium-denuded rings or in rings pretreated with L-NAME, a NO synthase blocker. There are three possible reasons for this effect. Firstly, NO, alone or combined with other unidentified substances produced in the endothelium, synergises with or facilitates the vasorelaxing effect of CO released from CORM-3. Secondly, CO released from CORM-3 induces release of NO from endothelial storage pools which in turn relaxes the aortic ring as formerly suggested in a study conducted on renal resistance arteries (Thorup *et al.*, 1999). However, the optimal concentration of CO needed to stimulate maximum release of NO in renal arteries was 10 nM, which is much lower than that liberated from 100 μM CORM-3, assuming that one mole of CO is released by each mole CORM-3 according to myoglobin assay (Clark *et al.*, 2003; Foresti *et al.*, 2004). Indeed, the higher the concentrations of CO applied to renal arteries, the less NO release was detected (Thorup *et al.*, 1999). Therefore, the vasorelaxing effect of CORM-3 is unlikely to be solely due to stimulation of NO release. Thirdly, NO formed in endothelium might form stable complexes with ruthenium, as previously suggested (Mosi *et al.,* 2002; Marmion *et al.,* 2004), and weaken the bond between carbonyl groups and the transition metal of CORM-3. As a result, CO is easily released from CORM-3 to induce vasorelaxation. The last hypothesis is the most relevant as ruthenium has a strong affinity for NO and has been proposed as a new therapeutic strategy to scavenge NO in some pathological conditions (Mosi *et al.,* 2002; Marmion *et al.,* 2004).

Another important finding in this study is that CORM-3 at concentrations as high as 500 μM had no notable toxic effect on aortic SMCs. This means that CORM-3 at concentrations that cause significant vasorelaxation (50-200 μM) is relatively safe to SMCs *in vitro.* This fact undoubtedly will encourage us to go further in our journey toward finding the optimal CORM-3 concentrations that can safely modulate vascular tone *in vivo*. At high concentration of 1000 μM both CORM-3 and iCORM-3 induced a significant loss in SMCs viability, this finding points towards the role of the chemical structure of CORM-3, particularly its heavy metal backbone, rather than released CO as mediating these toxic effects.

In conclusion, our study shows that CORM-3 delivers CO and exerts rapid endothelium-dependant vasorelaxation *ex vivo.* It appears that the sGC/cGMP pathway and NO produced from endothelium layer play roles in mediating CORM-3-induced vasorelaxation. In addition our study shows that CORM-3 at concentrations (50-200 μM) that induce significant vasorelaxation *ex vivo* had no notable toxic effect on aortic SMCs viability *in vitro.* Our findings support the idea that water-soluble metal carbonyls could be utilized as prototypic chemicals in the development of pharmacologically active compounds capable of delivering CO for the control of vascular functions and prevention of hypertension.

4 Vasoactive Properties of CORM-A1

Sodium boranocarbonate $Na_2[H_3BCO_2]$, which has been designated CORM-A1, is a newly identified water soluble carbon monoxide releasing molecule. Unlike CORM-3, CORM-A1 does not contain a transition metal but a boron atom to which a carboxyl group (COO^-) is covalently bound (Motterlini *et al.,* 2005) (**Figure 4.1A**). Another important difference between COM-A1 and CORM-3 is the kinetic of CO release from these water soluble molecules. While CORM-3 has been shown to release CO rapidly (within 1-5 min), CORM-A1 has a slower pattern of CO release at physiological pH ($t_{1/2}$ ~ 21 min) (Motterlini *et al.,* 2005).

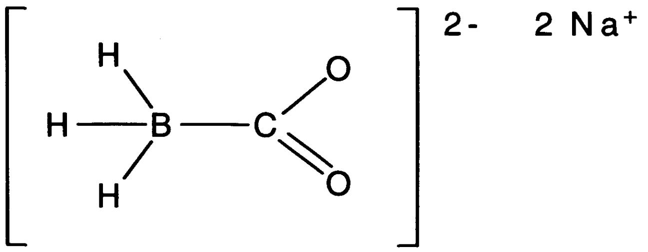


Figure 4.1A. Chemical structure of sodium boranocarbonate (CORM-A1) (adapted from Motterlini *et al.,* 2005).

In this project we studied the vasoactive properties of CORM-A1 and compared it to that elicited by CORM-3. For this purpose the aortic ring preparation model was used to assess the vasorelaxing properties of CORM-A1 on precontracted rings. Furthermore we dedicated a significant part of this study to explore underlying mechanisms that might mediate the vascular activities of CORM-A1. In particular we focused on the soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway as a potential cellular target. In addition the role of nitric oxide (NO) and the vascular endothelium layer in mediating CORM-A1 induced vasorelaxation was investigated. Finally we studied the effect of CORM-A1 on cultured aortic smooth muscle cell viability to rule out any toxic effect on smooth muscle cells.

4.1 Materials and Methods

4.1.1 Preparation of solutions

CORM-A1 and CORM-3 were prepared as previously described (Alberto *et al.*, 2001; Clark *et al.*, 2003). Stock solutions of CORM-A1 and CORM-3 were freshly prepared before the experiments by dissolving the compounds in distilled water. When assessed by a myoglobin assay or by an amperometric CO sensor, CORM-A1 has been shown to release CO slowly ($t_{1/2}$ ~ 21 min) compared to CORM-3 ($t_{1/2}$ 1-5 min) (Motterlini *et al.*, 2005). It has been noticed that acidic pH significantly accelerated the spontaneous release of CO from CORM-A1. Therefore we took advantage of this specific property of CORM-A1 and generated a CO-depleted inactive form (iCORM-A1) to be used as negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N_2 through the solution for 10 min in order to remove the residual CO gas. The solution of iCORM-A1, which is probably predominantly sodium borate, was finally adjusted to pH 7.4 and tested with the myoglobin assay to verify its inability to liberate CO (Alberto *et al.*, 2001; Motterlini *et al.*, 2005).

4.1.2 Aortic ring preparation

Transverse ring sections prepared from aortas of male adult Sprague Dawley rats (300-400 g) were suspended under 2 g tension in oxygenated Krebs Henseleit buffer as previously described in Materials and Methods (Chapter 2). To establish the potential vasorelaxant effects of CORM-A1, aortic rings were precontracted with phenylephrine (1 μM) before addition of the compound at different concentrations (40-160 μM). The extent of vasorelaxation over one hour was determined and compared with the effect produced by one addition of CORM-3 (100 μM). In another set of experiments, the inactive form (iCORM-A1) and sodium borohydride ($NaBH_4$) were used as negative control to exclude the possibility that vasorelaxation was a non-specific effect of boron compounds or borate. In addition myoglobin (80 μM) was added simultaneously with CORM-A1 (80 μM) to verify the role of CO in mediating the vasoactivity of CORM-A1. Because CO gas has been reported to activate guanylate cyclase to increase the production of cGMP (Hussain *et al.*, 1997; Nakatsu *et al.*, 2002), additional experiments were conducted to examine the involvement of cGMP in relaxation mediated by CORM-A1. For this, ODQ (30 μM), an inhibitor of guanylate cyclase, was added simultaneously with CORM-A1 to contracted aortic rings. The effect of CORM-A1 on vascular tone was also assessed in the presence of a stimulator of soluble guanylate cyclase, YC-1 (1 μM), which was added 30 min before phenylephrine. To investigate the possible involvement of NO in the relaxation elicited by CORM-A1, a separate group of experiments were performed by treating aortic rings with the NO synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) 30 min before addition of phenylephrine. CORM-A1 was also tested in endothelium-denuded rings. For this, the internal lumen of rings was subjected to gentle rubbing with a fine wooden stick and failure to dilate upon addition of acetylcholine was taken as a proof of successful endothelium removal.

4.1.3 Cell viability assay

Rat aortic smooth muscle cells (A7r5) were cultured as previously described in Materials and Methods (Chapter 2). Monolayers of A7r5 cells in 24 wells plates were incubated with CORM-A1 (10-1000 μM) solutions for 24 h. The effect of higher concentrations of CORM-A1 (100-1000 μM) were compared with similar concentrations of iCORM-A1. Then the cell viability was determined in aortic smooth muscle cells using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK) as previously explained in Material and Methods.

4.1.4 Statistical analysis

Vasodilatory responses were expressed as a percentage of the vasoconstriction induced by phenylephrine. Statistical analysis was performed using one-way and two-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P<0.05$.

4.2 Results

4.2.1 Vasodilatory effects of CORM-A1 in isolated aortic rings

Figure 4.1 shows the response of aortic rings pre-contracted with phenylephrine (Phe) to a single addition of CORM-3 (100 μM) or CORM-A1 (80 μM). As shown in this figure, CORM-3 induced rapid relaxation compared to CORM-A1 which caused a profound but more gradual reduction in the vascular tension. Specifically, CORM-A1 promoted a gradual relaxation which was maximal (96%) 30 min following administration of the molecule to the organ bath whereas CORM-3 elicited more than 50% relaxation within 5-10 min. Pre-contracted isolated vessels were also treated with increasing concentrations of CORM-A1 (40, 80 and 160 μM) and changes in vascular tone were recorded over one hour **(Figure 4.2)**. It can be observed that CORM-A1 elicited a significant relaxation over time in a concentration-dependent manner. For example, the extent of vasodilatation mediated by the three concentrations of CORM-A1 after 10 min was as follows: 21.0±2.3% with 40 μM CORM-A1, 40.2±3.4% with 80 μM CORM-A1 and 74.9±1.8% with 160 μM CORM-A1. The maximal relaxation of aortic rings was reached 30 min following addition of CORM-A1; the rings spontaneously re-contracted again thereafter but no vasorelaxation was observed if a new addition of CORM-A1 was added immediately after re-contraction (data not shown). The inactive form (iCORM-A1), which did not release CO, and sodium borohydride ($NaBH_4$), which was used as an additional negative control to exclude any effect of boron on vessel tone, failed to induce any relaxation of pre-contracted aortic rings **(Figure 4.3)**. Furthermore, the addition of myoglobin (80 μM) to the organ bath markedly reduced the vasorelaxation induced by CORM-A1 **(Figure 4.4)**. Taken together, the current results indicate that CO liberated from CORM-A1 is directly responsible for the observed vascular effect.

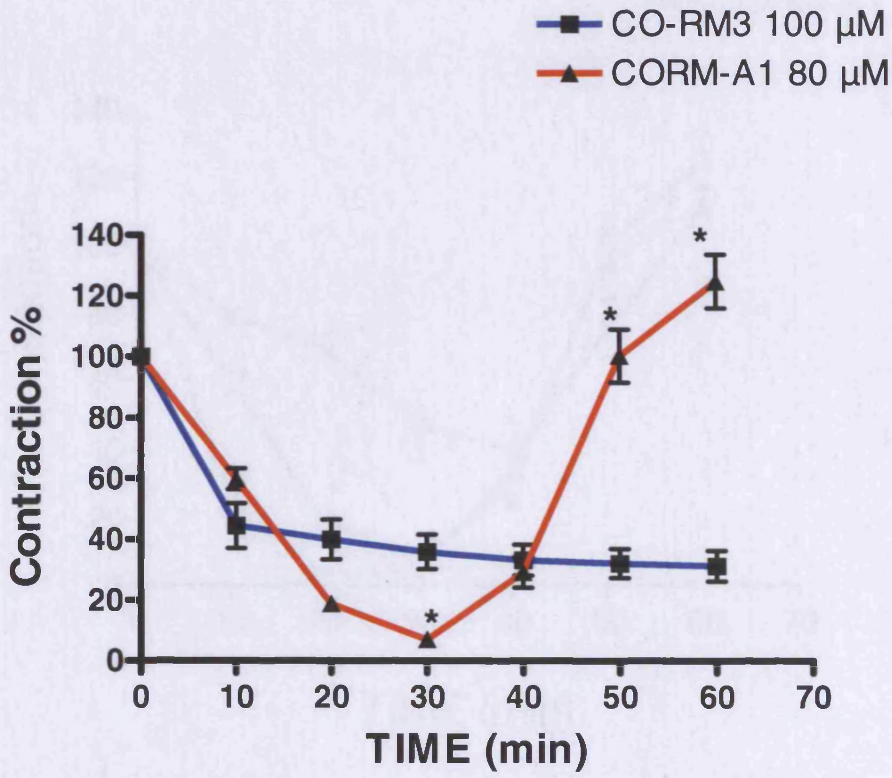


Figure 4.1. Effects of CORM-A1 and CORM-3 on vascular tone.

This graph compares the vasodilatory response of precontracted rings to one addition of CORM-3 (100 μM) to CORM-A1 (80 μM) over one hour. CORM-3 induced rapid vasorelaxation which reached a maximum within 10 min whereas CORM-A1 induced a gradual pattern of vasorelaxation that reached a maximum after 30 min then the aortic rings recontracted spontaneously. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-3 100 μM.

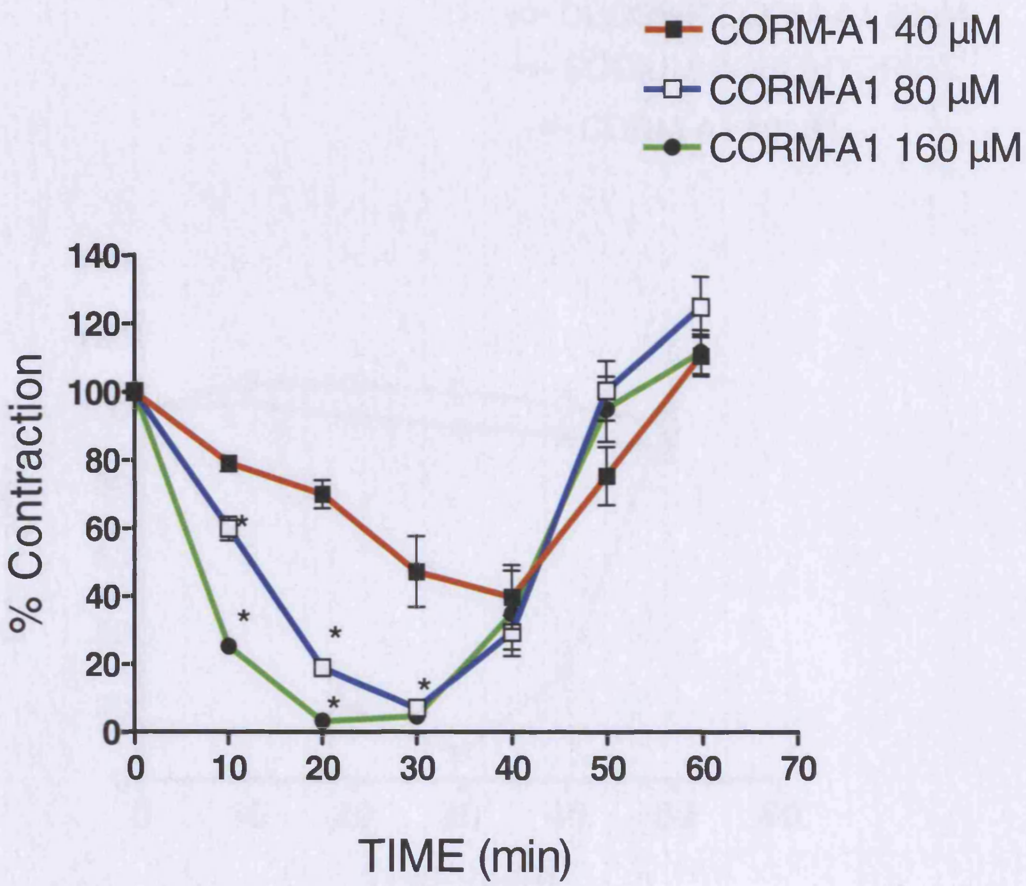


Figure 4.2. Effect of CORM-A1 on vascular tone.

This graph shows the vasodilatory response of precontracted aortic rings to three different concentrations of CORM-A1 (40, 80, and 160 µM). CORM-A1 caused significant relaxation over time in a concentrated-dependent manner. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-A1 40 µM.

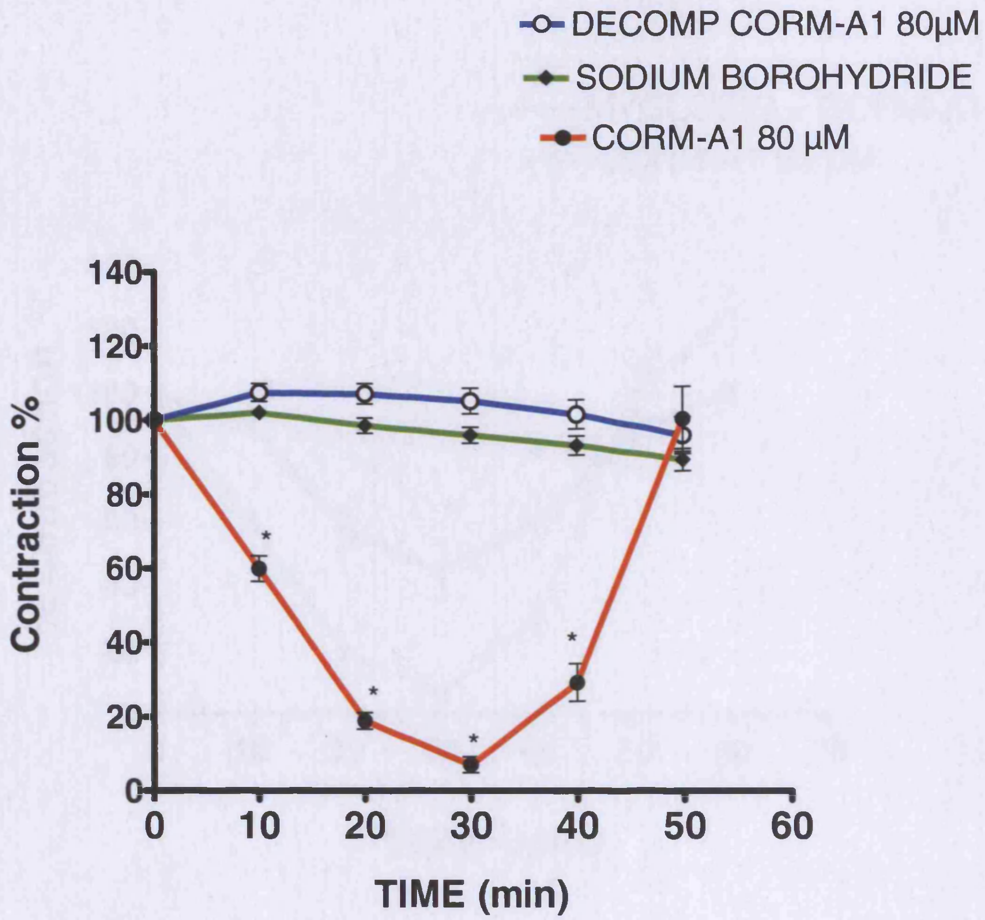


Figure 4.3. Effect of CORM-A1 on vascular tone

This graph shows the vasodilatory effects of 80 μM CORM-A1 on precontracted aortic rings compared to 80 μM iCORM-A1 and 80 μM sodium borohydride (NaBH4). CORM-A1 induced a gradual and profound vasorelaxation, whereas iCORM-A1 and NaBH4 were ineffective. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P<0.05 compared to decomposed CORM-A1 80μM.

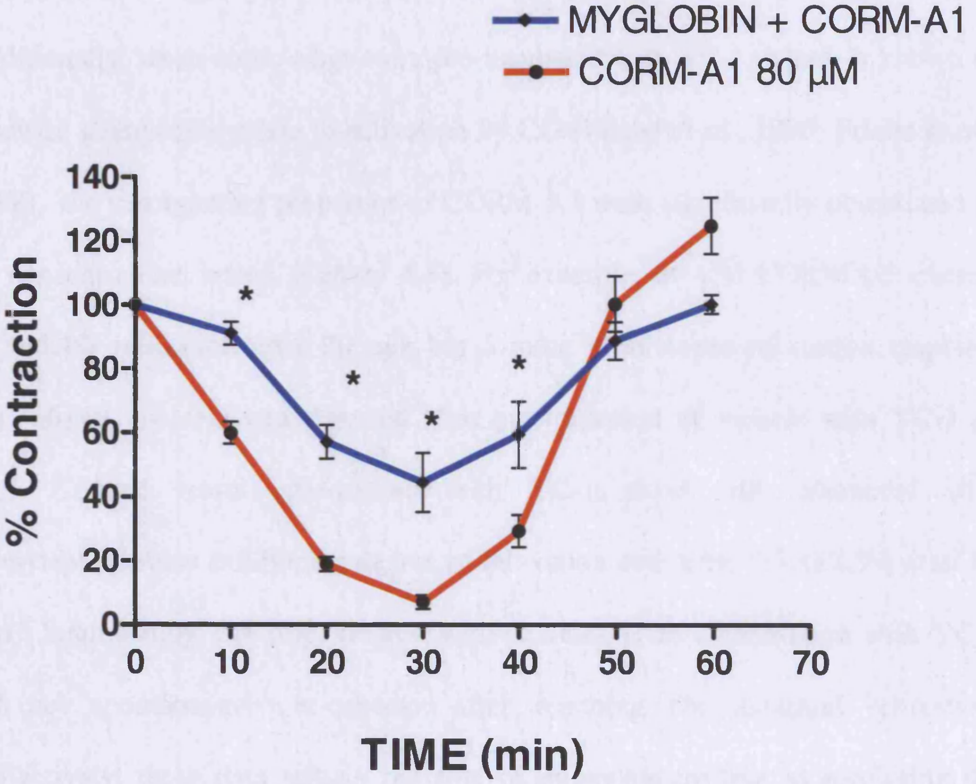


Figure 4.4. Myoglobin reduces CORM-A1 induced vasorelaxation.

Myoglobin 80 μM was added simultaneously with 80 μM CORM-A1 to precontracted isolated aortic rings. Myoglobin significantly reduced the CORM-A1-induced vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P< 0.05 compared to CORM-A1 80 μM.

4.2.2 CO-mediated vasorelaxation by CORM-A1: possible mechanisms of action

In this study, the vasoactivity of CORM-A1 was partially attenuated in the presence of a guanylate cyclase blocker (ODQ, 30 μM) **(Figure 4.5).** Additionally, when aortic rings were pre-incubated with YC-1, which is known to sensitize guanylate cyclase to activation by CO (Friebe *et al.*, 1996; Friebe *et al.*, 1998), the vasorelaxing properties of CORM-A1 were significantly potentiated at all concentrations tested **(Figure 4.6)**. For example, 20 μM CORMA-1 caused 15.4±5.1% relaxation after 30 min but a more pronounced relaxation response (66.7±6.0%, $p<0.01$) was detected after pre-treatment of vessels with YC-1 (1 μM). Control vessels pre-treated with YC-1 alone and contracted with phenylephrine also exhibited a degree of relaxation over time (25.8±2.5% after 30 min). Interestingly, the rings treated with CORM-A1 in combination with YC-1 did not spontaneously re-contract after reaching the maximal relaxation. Collectively, these data sustain the role of guanylate cyclase in mediating the vasorelaxing properties of CORM-A1.

CORM-A1 was also tested in endothelium-denuded rings to examine the contribution of endothelium in CORM-A1-mediated vasorelaxation **(Figure 4.7)**. Interestingly, absence of endothelium had no effect on CORM-A1 vasoactivity. Aortic rings were also pre-incubated with NO synthase inhibitor (L-NAME), to assess the involvement of NO in mediating the vasorelaxing properties of CORM-A1. Apart from an initial 17±2.4% reduction of the vasorelaxing effect of CORM-A1, in the first 10 min, the presence of L-NAME did not significantly change the response to CORM-A1 thereafter **(Figure 4.8)**. Taken together, these results suggest that NO and/or factors derived from the endothelium barely contribute to the vasodilatory response to CORM-A1.

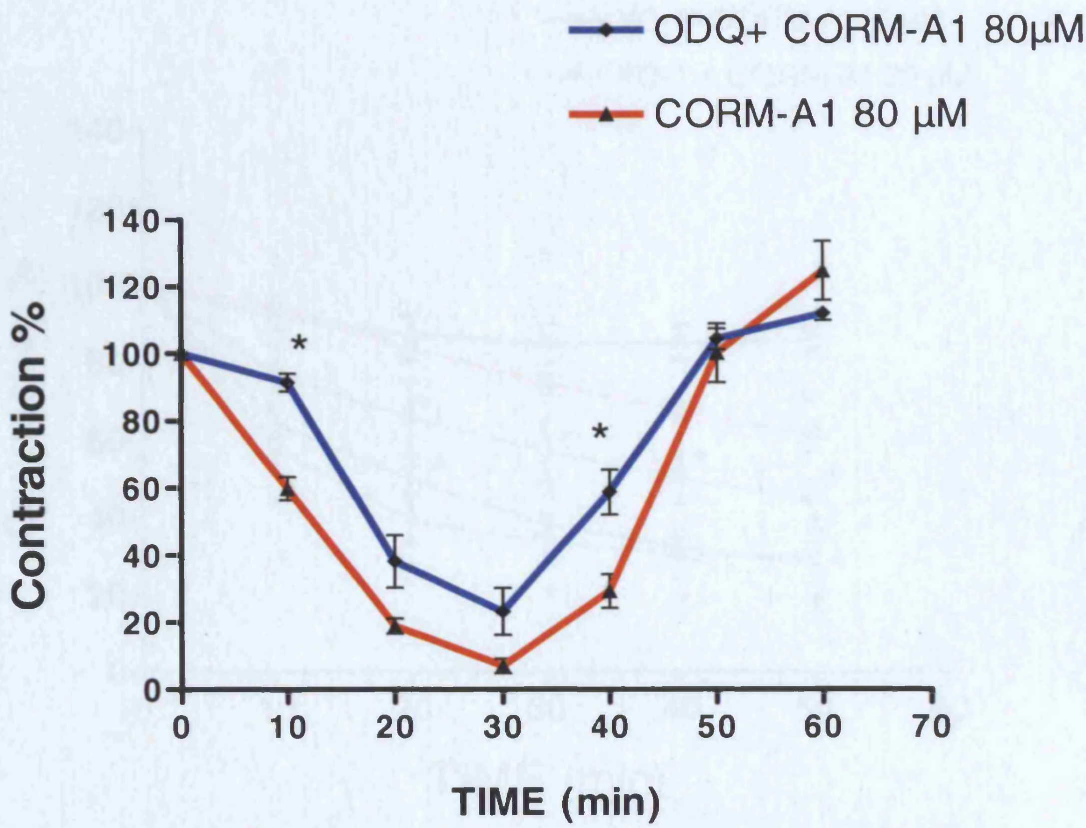


Figure 4.5. Inhibition of sGC pathway reduces CORMA-mediated vasorelaxation.

ODQ (30 μM) was added simultaneously with 80 μM CORM-A1 to precontracted isolated aortic rings. ODQ reduced the CORM-A1-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 10-12 independent experiments. *P<0.05 compared to CORM-A1 80 μM.

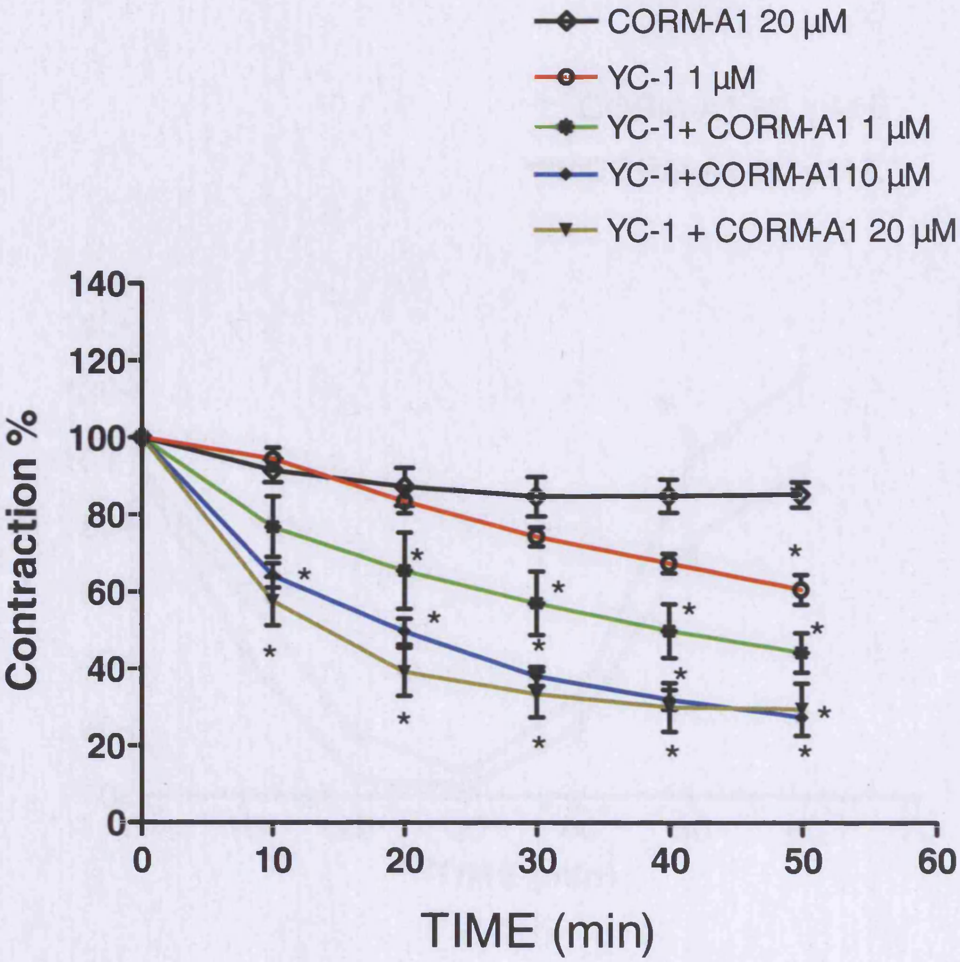


Figure 4.6. YC-1 potentiates CORM-A1-induced vasorelaxation.

Rings were incubated with 1 μM YC-1 30 min prior to addition of phenylephrine. Different concentrations of CORM-A1 (1, 10 and 20 μM) were added. YC-1 significantly potentiated the vasodilatory effects of all CORM-A1 concentrations. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-A1 20 μM.

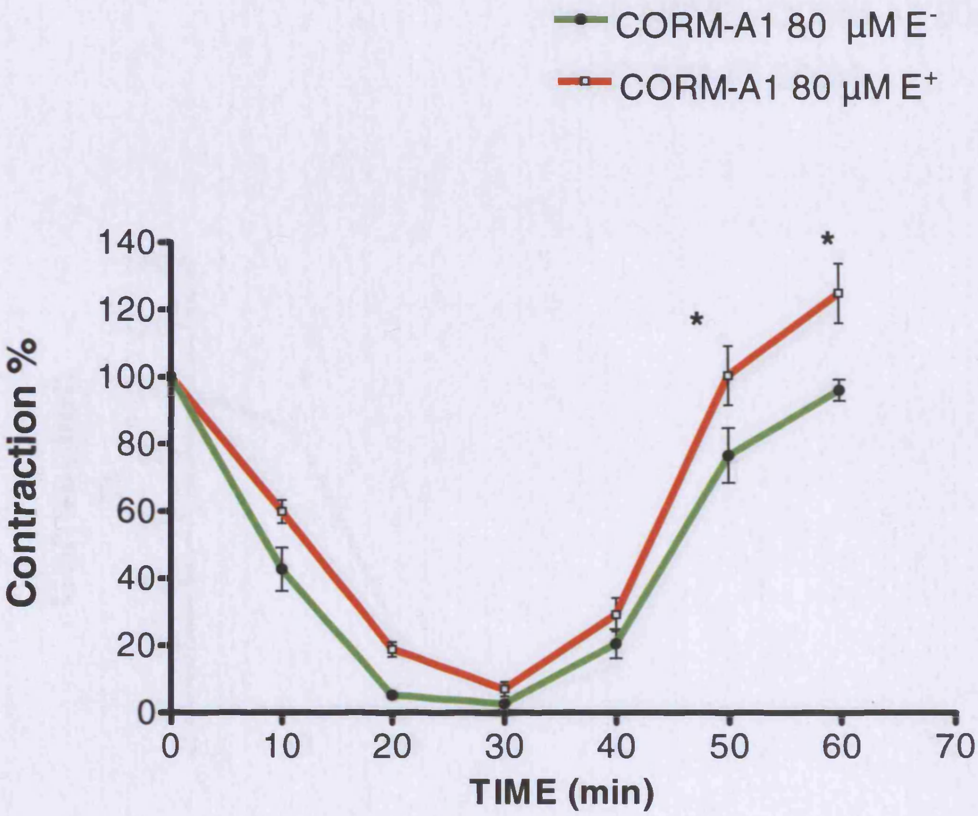


Figure 4.7. CORM-A1 induces endothelium independent vasorelaxation.

CORM-A1 (80 μM) was added to endothelium denuded aortic rings (E^-) and endothelium intact aortic rings (E^+). CORM-A1 induced endothelium-independent vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. $^*P<0.05$ compared to CORM-A1 80 μM E^+.

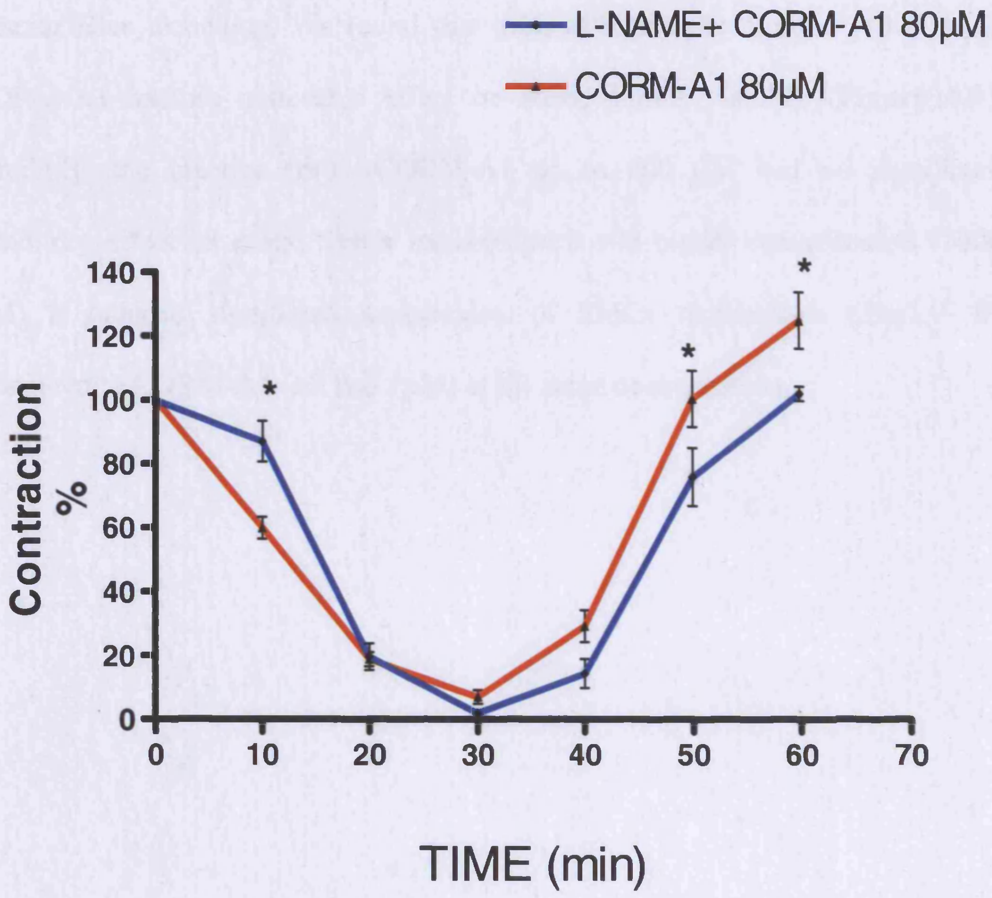


Figure 4.8. NO synthase inhibitor L-NAME does not affect CORM-A1-mediated vasorelaxation.

Aortic rings were incubated with 100 μM L-NAME 30 min prior to the addition of phenylephrine. Apart from mild inhibition at 10 min, L-NAME did not affect CORM-A1-induced vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-A1 80 μM alone.

4.2.3 Effect of CORM-A1 on SMCs viability

The effect of CORM-A1 on aortic SMCs (A7r5 cell line) was assessed using the Alamar Blue technique. We found that even at high concentration (1000 μM), CORM-A1 had no noticeable effect on aortic SMCs viability (**Figure 4.9**). Similarly, the inactive form iCORM-A1 up to 500 μM had no significant inhibitory effect on aortic SMCs metabolism but at higher concentration (1000 μM) it induced significant suppression of SMCs metabolism (39±2.9 %) compared to CORM-A1 (13.1±6.1μM) at the same concentration.

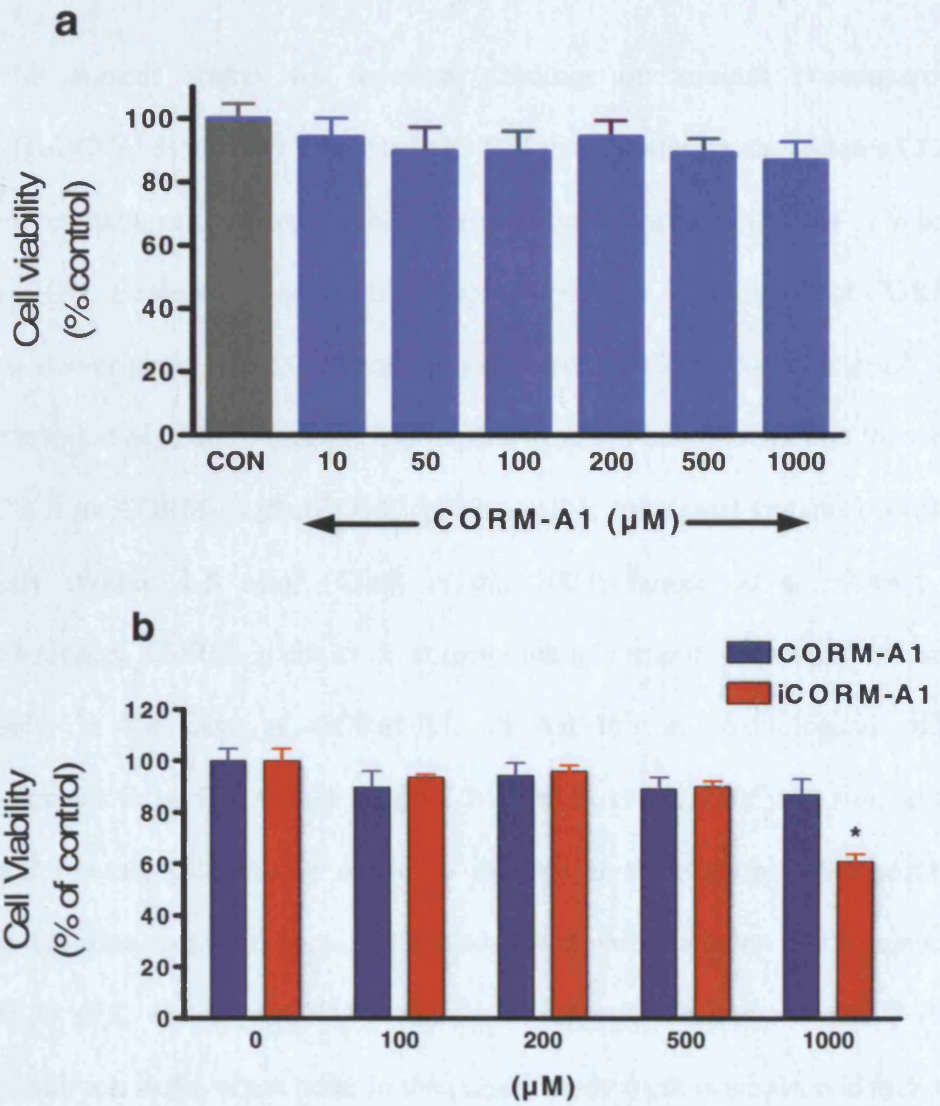


Figure 4.9. Effect of CORM-A1 on cell viability.

Aortic SMCs (A7r5) were treated with increasing concentrations of CORM-A1 (10-1000 μM) and iCORM-A1 (100-1000 μM) for 24 h and the change in cell viability was assessed using Alamar Blue technique. As shown in figure (a) CORM-A1 up to 1000 μM had no noticeable toxic effect on SMCs. Figure (b) shows that the inactive form iCORM-A1 up to 500 μM had no effect on cell viability but at 1000 μM it was more toxic than CORM-A1. Data represent the mean ± s.e.m. of 6-12 independent experiments. $^*P<0.05$ compared to control.

4.3 Discussion

In the current study, we represent findings on sodium boranocarbonate ($Na_2[H_3BCO_2]$ as a newly identified CO-RM that spontaneously releases CO with a different pattern compared to the ruthenium-based carbonyl CORM-3. When the rate of CO release was assessed by myoglobin assay it was found that CORM-A1 has a slower pattern of CO liberation compared to CORM-3 (Clark *et al.*, 2003; Motterlini *et al.*, 2005). Indeed, it is known from our recent work that the release of CO from CORM-3 ($[Ru(CO)_3Cl(glycinate)]$) in biological systems occurs very rapidly (within 1-5 min) (Clark *et al.*, 2003; Foresti *et al.*, 2004); as a consequence, CORM-3 elicits a correspondingly rapid relaxation in isolated vessels. In the case of CORM-A1, its half-life at physiological pH and temperature is approximately 21 min (Motterlini *et al.*, 2005). In line with this slower release, CORM-A-1 added to isolated aortic rings pre-contracted with phenylephrine promoted a gradual and sustained vasorelaxation that was maximal after 30 min, whereas CORM-3 caused a profound relaxation within 5-10 min after addition to the organ bath. In the current study there is ample evidence which points toward the role of CO in mediating the vasorelaxing properties of CORM-A1. Firstly, the inactive form, (iCORM-A1), which does not release CO when assessed by either myoglobin assay or CO sensor, could not elicit any vasorelaxation. Secondly, when precontracted aortic rings were treated with a single addition of $NaBH_4$ (80 μM), which was used as another negative control to verify any possible contribution of boron in the bioactivity mediated by CORM-A1 (Motterlini *et al.*, 2005), no significant change in vascular tone was detected. Thirdly, myoglobin, a CO scavenger, significantly reduced the extent of vasodilatation exerted by CORM-A1, confirming the direct involvement of CO in the observed biological effects.

An interesting finding in our result is the recontraction that followed CORM-A1-induced relaxation in aortic rings. Almost all aortic rings treated with CORM-A1 (40-160 μM) elicited spontaneous significant recontraction after around 40 min from adding CORM-A1 to the organ bath. The mechanisms underlying this contraction are not obvious and possible explanations include: (1) CORM-A1 compound might have intrinsic vasoconstrictor property but CO-induced vasorelaxation suppresses this effect and once the CO effect disappears from the system this vasoconstrictive mechanism re-asserts itself; (2) the slow pattern of CO release from CORM-A1 might activate other vasoconstriction mechanisms which start to elicit their actions after 40 min from adding CORM-A1 to organ bath or when the CO effect is completed; (3) it might simply be physical recoil of aortic rings as a response to the gradual vasorelaxation. In preliminary experiments CORM-A1 (80 μM) failed to induce relaxation in spontaneously recontracted aortic rings previously treated with the same concentration of CORM-A1. Interestingly, higher concentration of CORM-A1 (160 μM) or another vasorelaxing factor (CORM-3 100 μM) was needed to elicit vasorelaxation. The possible explanation for this observation is that either CO RM-A1 or its released CO might desensitize the cellular receptors that mediate the vascular activities of CORM-A1, therefore higher concentrations or other vasodilators are needed to activate such receptors.

The presence of YC-1, which is a well-known benzylindazole derivative that activates the soluble guanylate cyclase (sGC)/cGMP pathway, markedly amplified the extent of vasodilatation caused by CORM-A1 (at concentrations as low as 1 μM) in isolated aortas. Furthermore, the sGC inhibitor (ODQ), mildly reversed the vasorelaxation of aortic rings induced by CORM-A1. Taken together, these findings indicate that sGC played a role in mediating the vasorelaxing properties of CORM-A1. Interestingly, CORM-A1 in combination with YC-1 did not result in recontraction as opposed to CORM-A1 alone. YC-1 might prolong the pharmacological action of CORM-A1 or influence vasoconstrictor mechanisms. Therefore, we believe that, like CORM-3, a combination of CORM-A1 and YC-1 might have important therapeutic applications in vascular diseases. A remarkable finding of the current study is that absence of endothelium or inhibition of NO synthase activity by L-NAME, failed to prevent the vasorelaxing effects of CORM-A1. These results suggest that the pharmacological action of CO released from CORM-A1 is independent of the endothelial function.

Another important finding of this study is that CORM-A1 up to concentrations as high as 1000 μM had no noticeable negative effect on aortic SMCs viability. At 1000 μM CORM-A1 induced only 13.1±6.1 % suppression of cell metabolism compared to 43.9±6.1 % induced by CORM-3. The relative safety of CORM-A1 on cultured cells will undoubtedly encourage us to go further in our long journey to design effective carbon monoxide releasing molecules that can be used safely on *in vitro*, *ex vivo* and *in vivo* experimental models. The current results show that up to 500 μM, the inactive form (iCORM-A1) had similar effect on SMCs viability to that elicited by its active form but at higher concentration iCORM-A1 induced more suppression on cell metabolism (29.0±2.8 %) than CORM-A1 (13.1±6.1 %). This result does not accord with the result obtained with CORM-3 where the active form was more toxic than the inactive form at higher concentrations. The difference in kinetics of CO release from these CO-RMs might explain the discrepancy between their effects on SMCs viability at high concentration.

To sum up, the current results show that the water soluble sodium boranocarbonate molecule (CORM-A1), promotes gradual endothelium-independent vasorelaxation which reflects its gradual pattern of CO release compared to CORM-3. This vasorelaxation is partially mediated by activation of soluble guanylate cyclase and it appears that NO has little or no role in mediating such effect. In addition, CORM-A1 even at high concentrations has no remarkable inhibitory effect on cultured aortic SMCs viability. Our data highlight the effect of CO release kinetics from different CO-RMs on their pharmacological actions. Taken together, these data show that CORM-A1 is another example of carbon monoxide releasing molecules that can be further modified to be utilized as pharmacologically active compounds capable of delivering CO for the control of vascular functions and prevention of hypertension.

5 The role of K^+ channels in regulating vasorelaxing properties of water-soluble carbon monoxide releasing molecules, CORM-3 and CORM-A1.

Potassium (K^+) channels are groups of integral membrane proteins that selectively transport K^+ across the cell membrane (Korn and Trapani, 2005). They have been found in all mammalian cells and have diverse functions in both excitable and non excitable cells. In fact K^+ channels are more sophisticated than any other ion channel super family found in mammalian cells (Cao *et al.,* 2002). In arterial smooth muscle cells almost every physiological vasoconstrictor and vasodilator has been shown to modulate one type or another of K^+ channels (Standen and Quayle, 1998). In general most vasoconstrictors and vasodilators have multiple pathways of action and among these pathways, K^+ channels contribute to regulate membrane potential and vascular contractile tone (Standen and Quayle, 1998). It is well known that activation of K^+ channels in arterial smooth muscle cells (SMCs) induces vasodilatation and lowers blood pressure, whereas inhibition of these channels leads to vasoconstriction (Nelson and Quayle 1995). At least four K^+ channels subgroups have been identified in vascular SMCs (Nelson and Quayle 1995; Standen and Quayle 1998; Korovkina and England 2002b; Jackson *et al.,* 2005): The first one is Ca^{+2} activated K^+ (K_{Ca}) channels which respond to change in intracellular Ca^{+2} to regulate intrinsic tone in small arteries. It has been proposed that any rise of intravascular pressure through membrane depolarization and elevation of intracellular calcium activates Kca channels. Activation of K_{Ca} leads to K^+ efflux, which counteract membrane depolarization and vasoconstriction (Brayden and Nelson 1992). K_{Ca} channels are divided into three groups: big conductance (BK_{Ca}), intermediate conductance

(IK_{Ca}) and small conductance (SK_{Ca}) channels. The second K^+ channels subgroup is voltage dependant K^+ (Kv) channels which respond to depolarization stimuli. Activation of Kv channels causes efflux of K^+ ions, which leads to membrane hyperpolarization and maintains the resting membrane potential at -40 to - 60 mV under physiological situations (Cao *et al.,* 2002). The third subgroup is inward rectifier K^+ (Kir) channels which appear to mediate external K^+-induced hyperpolarization and dilatation of resistance arteries (Nelson and Quayle, 1995). Finally the fourth subgroup is ATP sensitive K^+ (K_{ATP}) channels which is sensitive to some molecules produced from cellular energy metabolism. Among these molecules, nucleotide phosphates (ATP and ADP) are the most common (Zhuo *et al.*, 2005). The decrease of intracellular ATP leads to opening of the K_{ATP} channels, which will induce vasodilatation and increase blood flow into organs. In vascular SMCs the most abundant K^+ channels are BK_{Ca} and Kv channels (Korovkina and England, 2002b).

The role of K^+ channels in endothelial cells (EC) is not as well understood as that in SMCs. K_{Ca}, Kv, K_{ATP} and Kir channels have all been identified in endothelial cells (Coleman *et al.*, 2004). In contrast to SMCs, IK_{Ca} and SK_{Ca} are abundant in EC and it appears that these channels play essential roles in production of endothelium-derived hyperpolarizing factor (EDHF) which travels through myoendothelial gap junctions to SMCs to induce hyperpolarization and vasorelaxation (Coleman *et al.*, 2004).

Among different K^+ channel subgroups in vascular SMCs, K_{Ca} channel subgroups have been supposed to be mediators for carbon monoxide (CO)-induced vasorelaxation (Wang *et al.*, 1997b; Zhang *et al.*, 2001a; Wang and Wu 2003; Xi *et al.*, 2004). CO appears to modify BK_{Ca} directly by increasing its sensitivity to calcium (Ca^{2+}) ions. In coronary artery, chronic CO exposure has been found to enhance 4-aminopyridine (4-AP) current in isolated coronary myocytes and because 4-AP, at low concentration, is a general blocker of K_V channels the effect of CO on K_V was suggested (Barbe *et al.,* 2002b).

The present study was designed to investigate the potential role of different K^+ subfamilies in mediating CORMA1 and CORM-3-induced vasorelaxation. For this purpose aortic rings were pre-treated with some known K^+ channel inhibitors before adding CO-RMs and the vascular tone was measured using aortic ring preparation model.

5.1 Material and Methods

5.1.1 Aortic ring preparation

Transverse sections of aortic rings have been prepared as previously described in Materials and Methods (Chapter 2). To establish the role of K^+ channel subgroups in mediating CORM-A1 and CORM-3-induced vasorelaxation, the aortic rings were divided into six groups. Each group was treated with different K^+ channel subgroup inhibitors for 30-60 min before contracting the rings with phenylephrine (1-1.5 μM). The response of contracted rings to a single addition of CORM-A1 (80 μM) or three consecutive additions of CORM-3 (100 μM) was then measured over time as described before in Materials and Methods (Chapter 2). The first group was incubated with 30 mM tetraethyammonium (TEA), a nonslective K^+ channel inhibitor, for 60 min. The second group of rings were pretreated with 10 μM glibenclamide (GLI), a K_{ATP} channel inhibitor, for 30 min. The third group was incubated with 1 mM of 4-aminopyridine (4-AP), a K_V channel inhibitor, for 30 min. The fourth group of rings was pretreated with 100 nM charybdotoxin (CHB), a BK_{Ca} channel inhibitor, for 60 min. The fifth group was incubated with 100 nM apamine (APA), a SK_{Ca} for 60 min. Finally the sixth group was incubated with both apamine (100 nM) and charybdotoxin (100 nM) simultaneously for 60 min before contract the rings with phenylephrine.

In another set of experiments the ability of CORM-A1 and CORM-3 to induce vasorelaxation in high KCl contracted rings was assessed. For this purpose a group of aortic rings was contracted with high KCl (110 mM) solution then the response of these rings to single addition of CORM-A1 (80 μM) or three addition of CORM-3 (100 μM) was measured over time.

5.1.2 Statistical analysis

Vasodilatory responses were expressed as a percentage of the vasoconstriction induced by phenylephrine or KCl. Statistical analysis was performed using two-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P<0.05$.

5.2 Results

5.2.1 Effect of potassium channel blockers on CO-RMs-induced vasorelaxation

TEA is a non selective inhibitor of most K^+ channel subgroups. At 10 mM concentration which was reported to inhibit K_{ATP}, K_V, K_{Ca} channels (Qiu and Quilley 2001; Nelson and Quayle 1995) TEA failed to affect the vasorelaxing properties of CORM-3 (preliminary results). However, at 30 μM it completely reversed the CORM-3-induced vasorelaxation of phenylephrine contracted rings **(Figure 5.1a)** and also significantly attenuated CORM-A1-induced vasorelaxation **(Figure 5.1b).** These findings indicate that K^+ channels play a role in mediating the vasorelaxing effect of CORM-3 and CORM-A1.

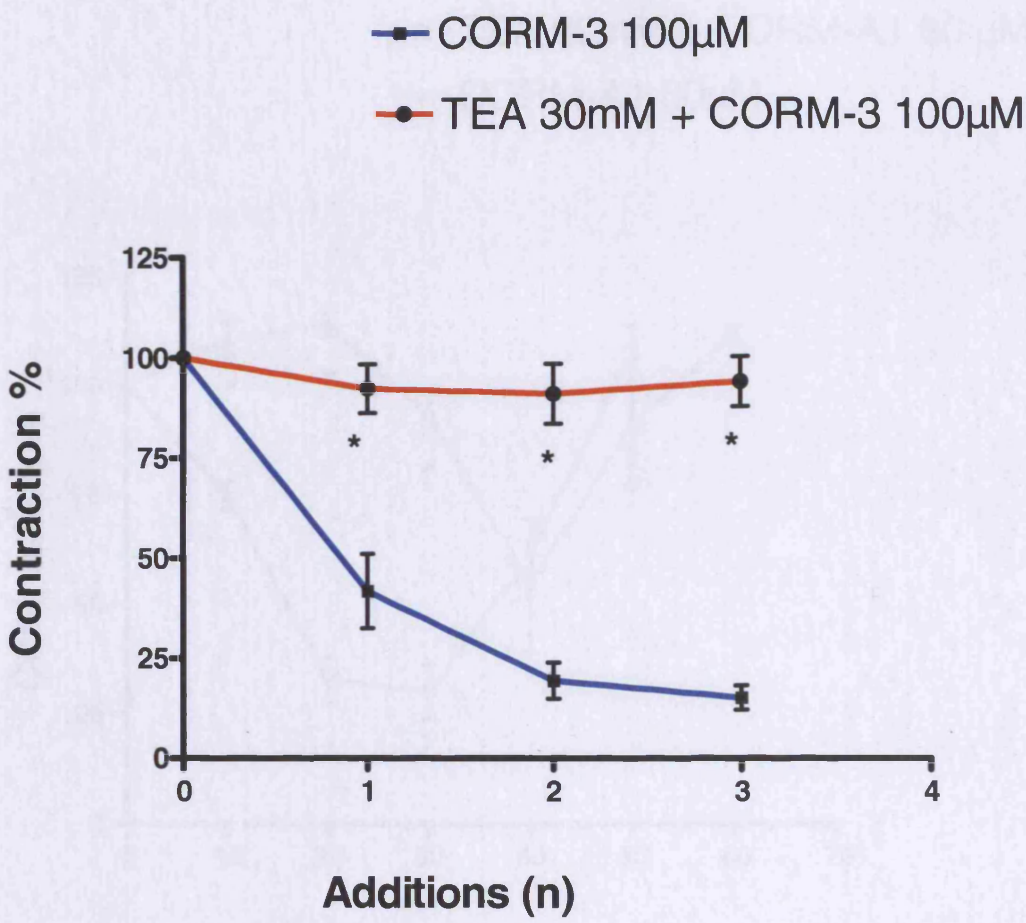


Figure 5.1A. Nonselective K+ channel blocker, tetraethylammonium (TEA), reversed CORM-3-mediated vasorelaxation.

Rings were incubated with 30 mM tetraethylammonium (TEA) 60 min prior to the addition of phenylephrine. TEA completely reversed CORM-3-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-3 (100 μM) alone.

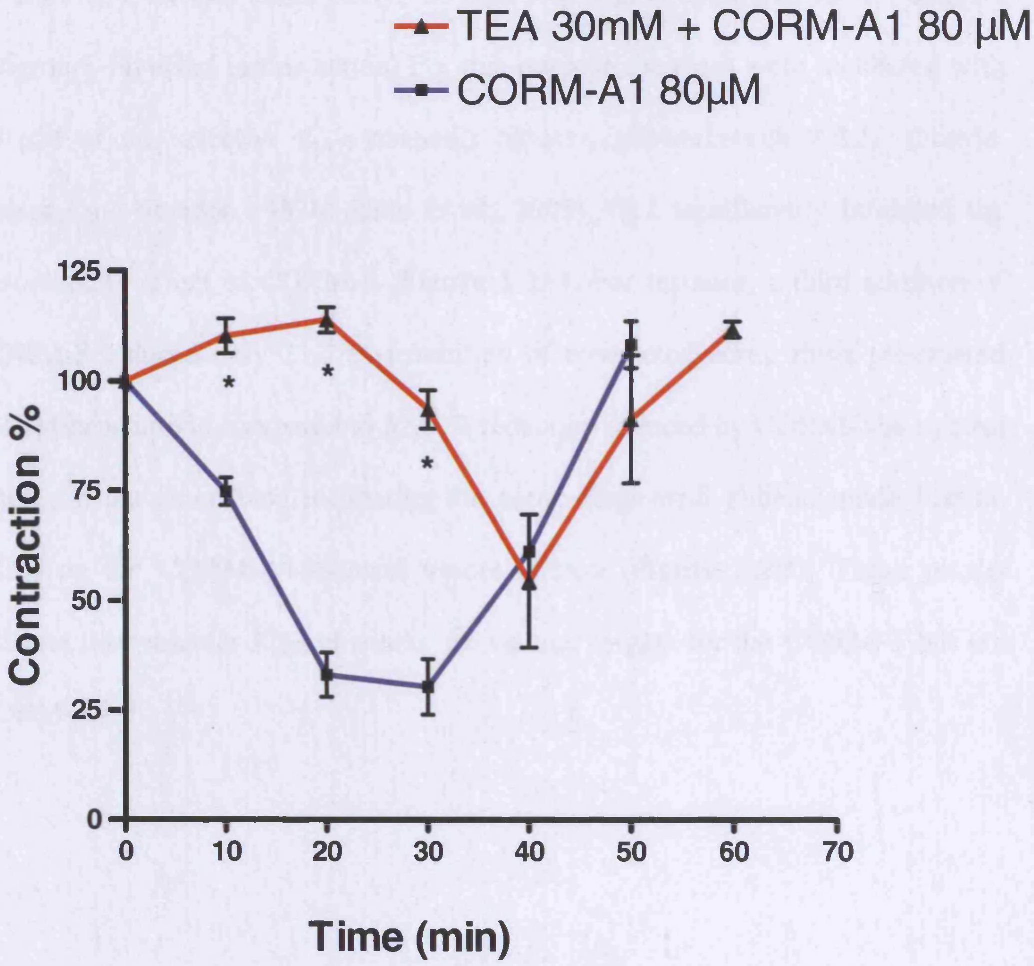


Figure 5.1B. Nonselective K+ channel blocker, tetraethylammonium(TEA), attenuated CORMA1-mediated vasorelaxation.

Rings were incubated with 30 mM tetraethylammonium (TEA) 60 min prior to the addition of phenylephrine. TEA significantly attenuated CORM-A1-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORMA1 (80 µM) alone.

5.2.2 *Effect of K_{ATP} channel inhibitor on CO-RMs induced vasorelaxation.*

After establishing the role of K^+ channels in mediating the vasorelaxation induced by both the CO-RMs under study, the next step was to determine the K^+ channel subgroups involved in this action. For this purpose the rings were incubated with 10 μM of the selective K_{ATP} channels blocker, glibenclamide (GLI) (Quayle, Nelson, and Standen 1997b; Zhuo *et al.*, 2005). GLI significantly inhibited the vasorelaxing effect of CORM-3 **(Figure 5.2A).** For instance, a third addition of CORM-3 induced only 21.5 % relaxation of contracted aortic rings pre-treated with glibenclamide compared to 52.7 % reduction induced by CORM-3 in control rings. On the other hand incubating the aortic rings with glibenclamide had no effect on the CORM-A1-induced vasorelaxation **(Figure 5.2B).** These results indicate that vascular K_{ATP} channels are cellular targets for the CORM-3 but not CORM-A1.

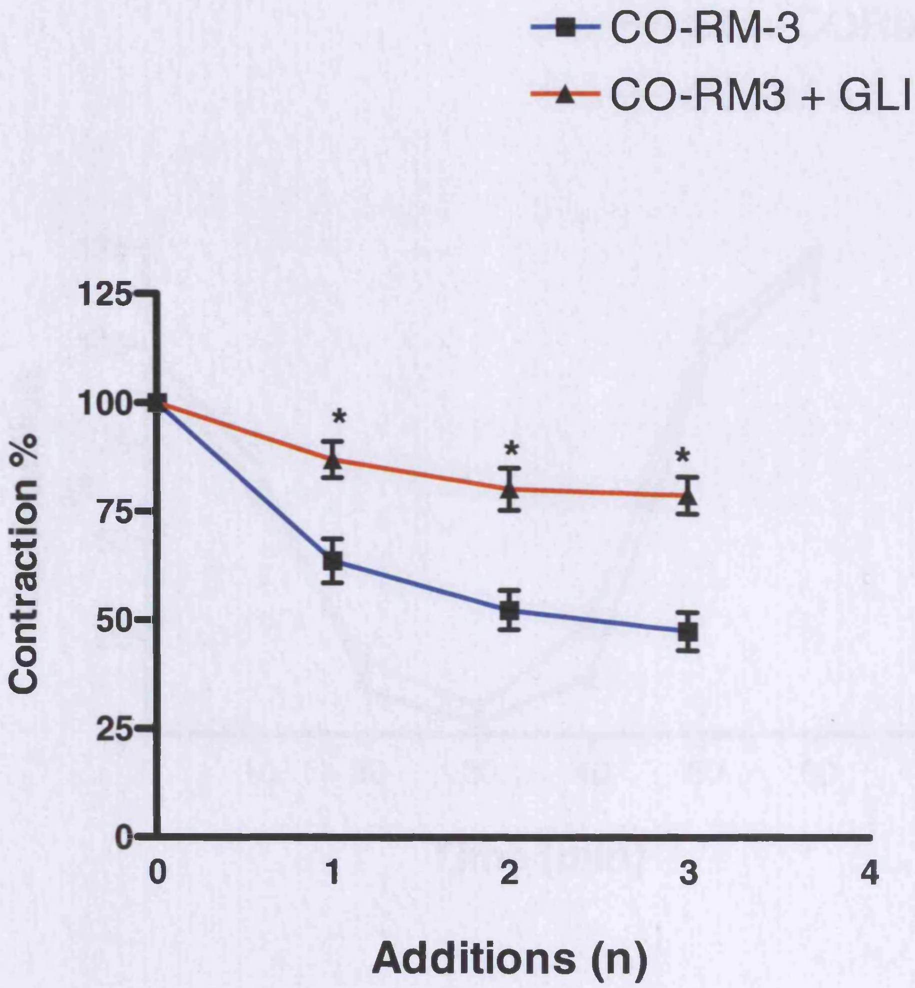


Figure 5.2A. K_{ATP} channel blocker, glibenclamide, attenuated CORM-3-mediated vasorelaxation.

Rings were incubated with 10 μM glibenclamide (GLI) 30 min prior to the addition of phenylephrine. Glibenclamide attenuated CORM-3-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-3 (100 μM) alone.

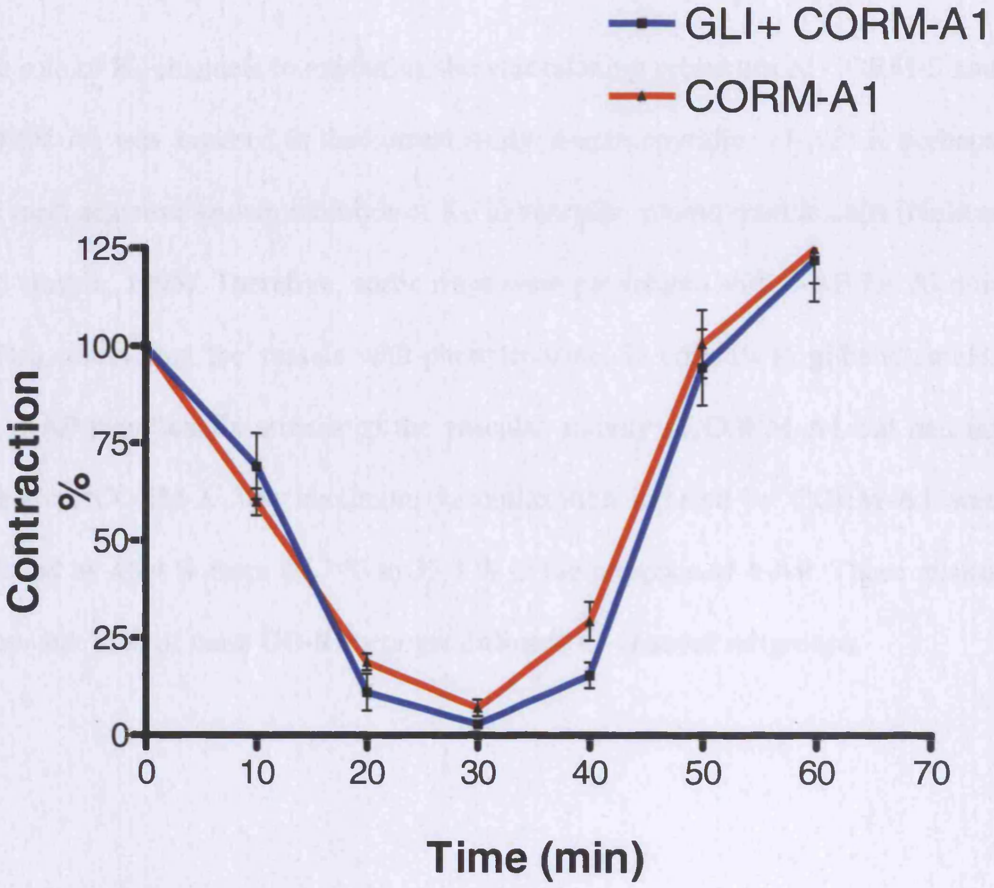


Figure 5.2B. K_{ATP} channel blocker, glibenclamide, did not affect CORM-A1-mediated vasorelaxation.

Rings were incubated with 10 μM glibenclamide (GLI) 30 min prior to the addition of phenylephrine. Glibenclamide did not affect CORM-A1-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-A1 (80 μM) alone.

5.2.3 The role of K_V channels in *mediating* the vasorelaxing properties of CO-RMs.

The role of K_V channels in mediating the vasorelaxing properties of CORM-3 and CORM-A1 was assessed in the current study. 4-aminopyridine (4-AP) is perhaps the most selective known inhibitor of K_V in vascular smooth muscle cells (Nelson and Quayle, 1995). Therefore, aortic rings were pre-treated with 4-AP for 30 min before contracting the vessels with phenylephrine. In contrast to glibenclamide, the 4-AP significantly attenuated the vascular activity of CORM-A1 but had no effect on CORM-3. The maximum vasorelaxation induced by CORM-A1 was reduced by 45.4 % from 80.7 % to 35.3 % in the presence of 4-AP. These results show that each of these CO-RMs target different K^+ channel subgroups.

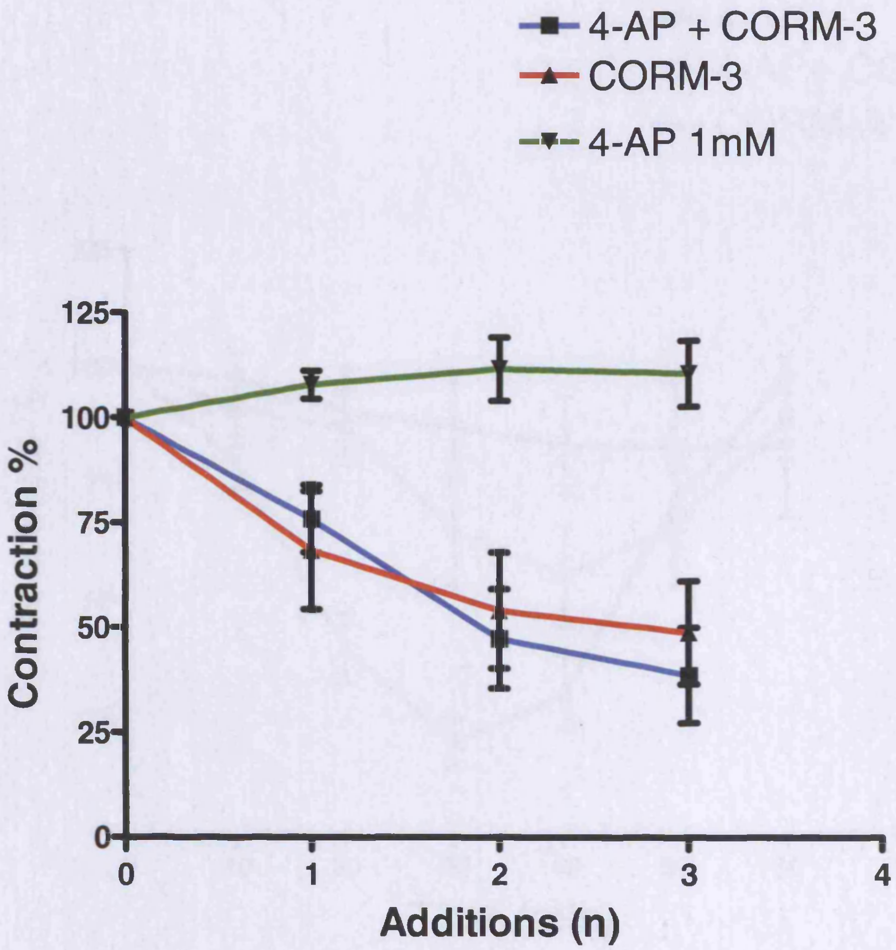


Figure 5.3A. K_V channel blocker, 4-aminopyrydine, did not affect CORM-3-mediated vasorelaxation.

Rings were incubated with 1mM 4-aminopyridine (4-AP) 30 min prior to the addition of phenylephrine. 4-AP did not affect CORM-3-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^*P<0.05$ compared to CORM-3 (100 μM) alone.

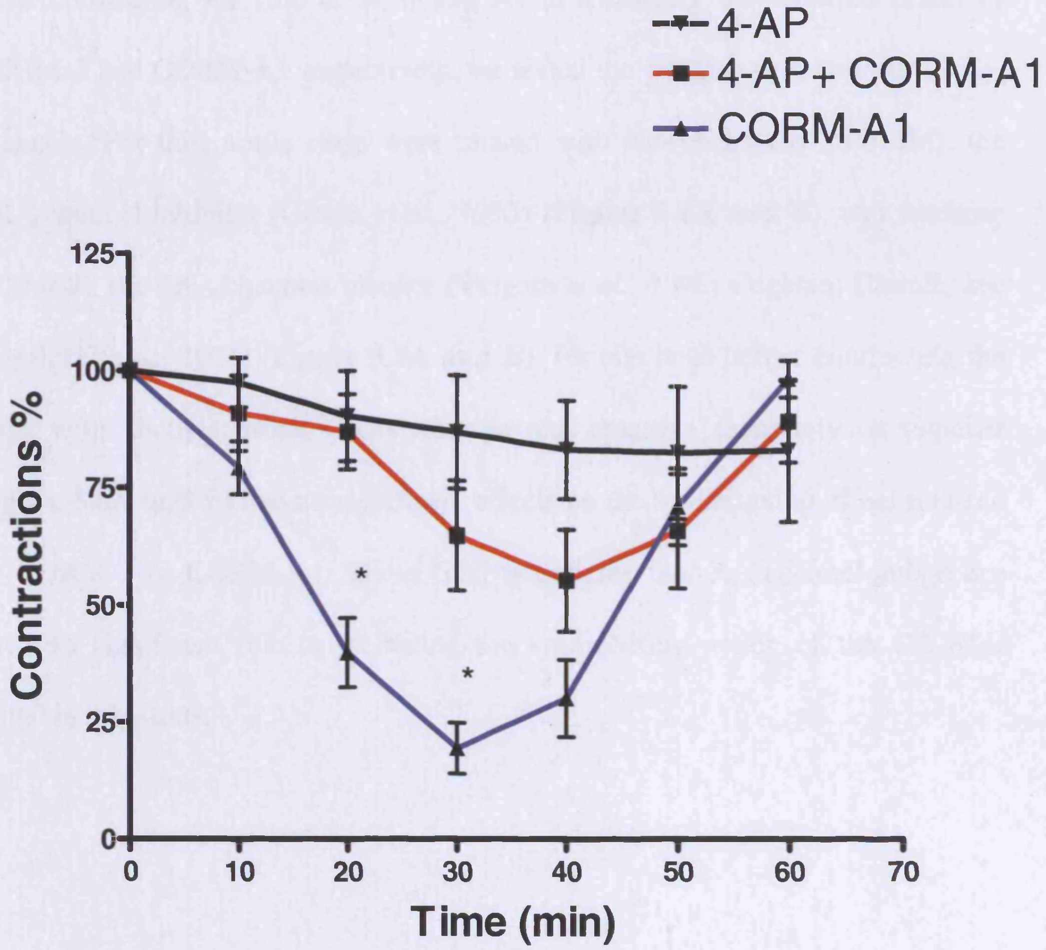


Figure 5.3B. K_V channel blocker, 4-aminopyrydine, reduced CORM-A1-mediated vasorelaxation.

Rings were incubated with 1mM 4-aminopyridine (4-AP) 30 min prior to the addition of phenylephrine. 4-AP significantly reduced CORM-A1-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^*P<0.05$ compared to CORM-A1 (80 μM) alone.

5.2.4 The role of K_{Ca} channels in mediating the vasorelaxing properties of CO-RMs

After confirming the role of K_{ATP} and K_V in mediating the vascular effect of CORM-3 and CORM-A1 respectively, we tested the possible involvement of K_{Ca} channels. For this, aortic rings were treated with charybdotoxin (100 nM), the BK_{Ca} channel inhibitor (Garcia *et al.*, 1995) **(Figure 5.4A and B)**, and apamine (100 nM), the SK_{Ca} channels blocker (Vergara *et al.*, 1998; Coghlan, Carroll, and Gopalakrishnan 2001) **(Figure 5.5A and B)**, for one hour before contracting the rings with phenylephrine. Charybdotoxin and apamine separately or together **(figure 5.6A and B)** had no significant effects on the vasorelaxing effect induced by CORM-3 or CORM-A1. These finding indicate that K_{Ca} channel subgroups have no significant role in mediating the vasorelaxing action of the CO-RMs tested in this study.

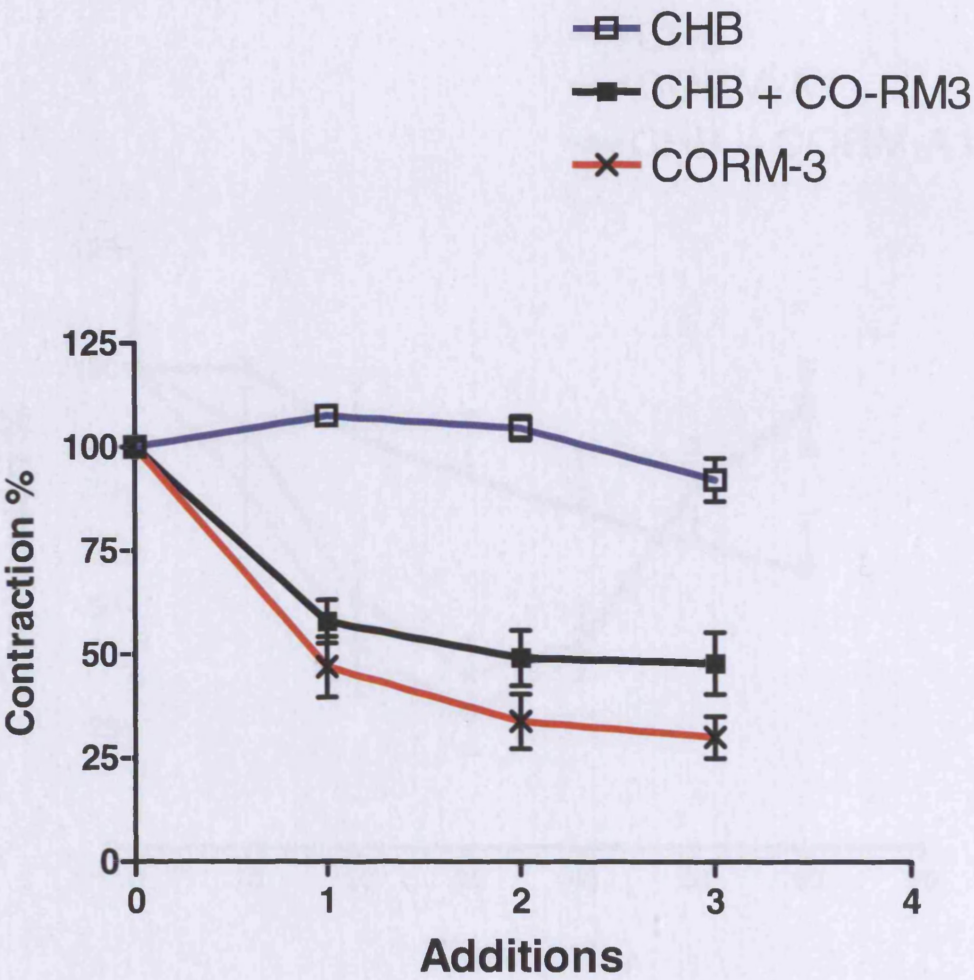


Figure 5.4A. BK_{Ca} channel blocker, charybdotoxin, mildly attenuated CORM-3-mediated vasorelaxation.

Rings were incubated with 100 nM charybdotoxin (CHB) 60 min prior to the addition of phenylephrine. CHB elicited mild effect on CORM-3-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-3 (100 μM) alone.

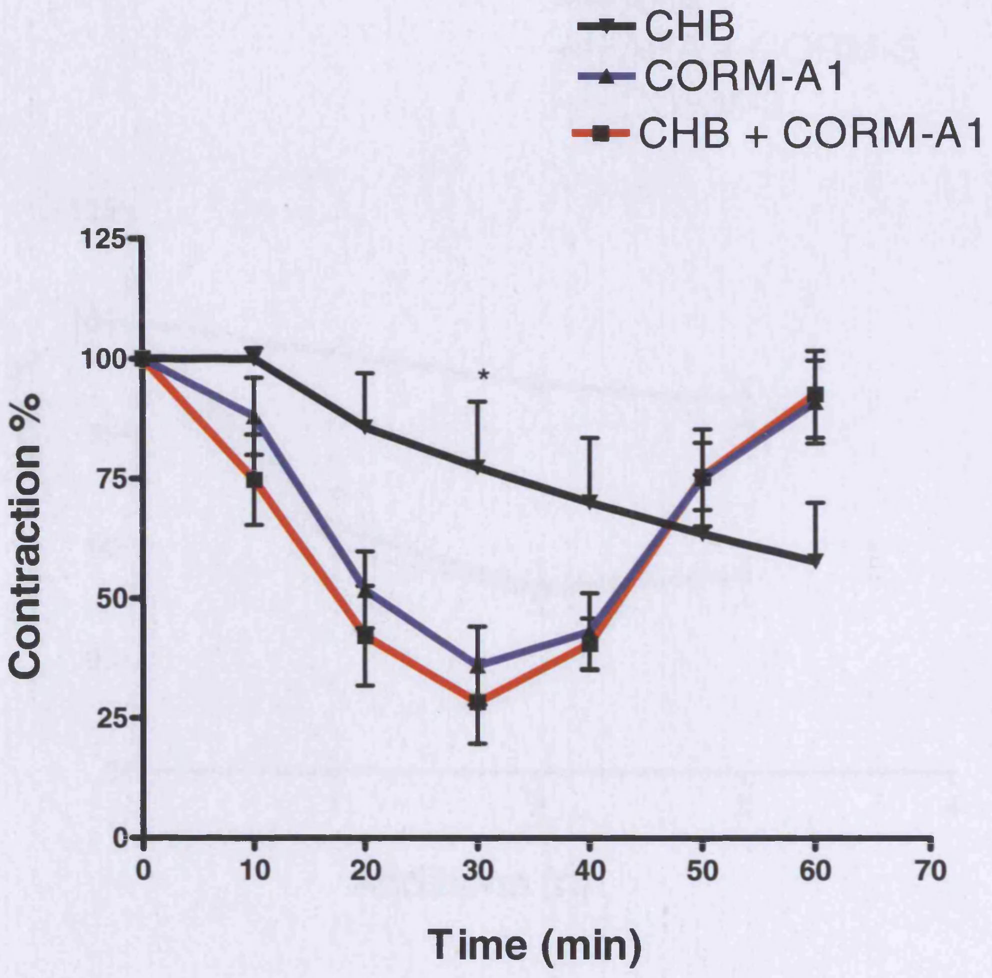


Figure 5.4B. BK_{Ca} channel blocker, charybdotoxin, did not affect CORM-A1-mediated vasorelaxation.

Rings were incubated with 100 nM charybdotoxin (CHB) 60 min prior to the addition of phenylephrine. CHB did not affect CORM-A1-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-A1 (80 μM) alone.

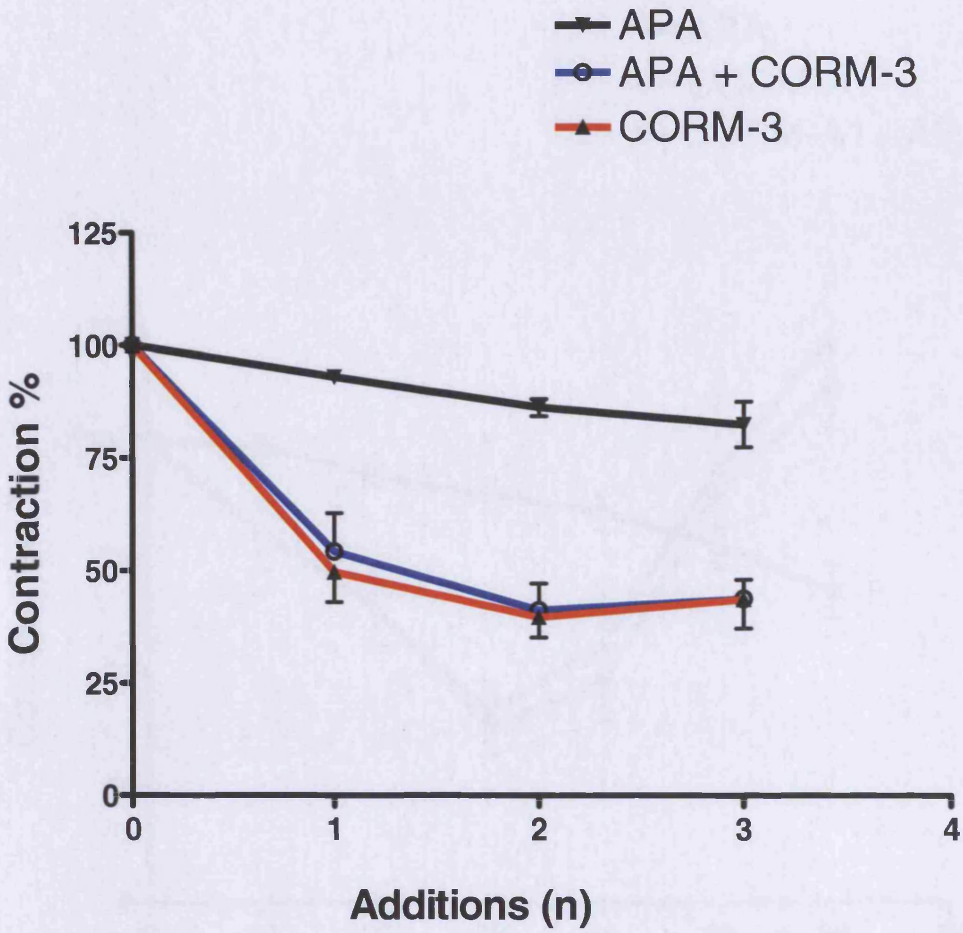


Figure 5.5A. SK_{Ca} channel blocker, apamine, did not affect CORM-3-mediated vasorelaxation.

Rings were incubated with 100 nM apamine (APA) 60 min prior to the addition of phenylephrine. APA did not affect CORM-3-mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-3 (100 μM) alone.

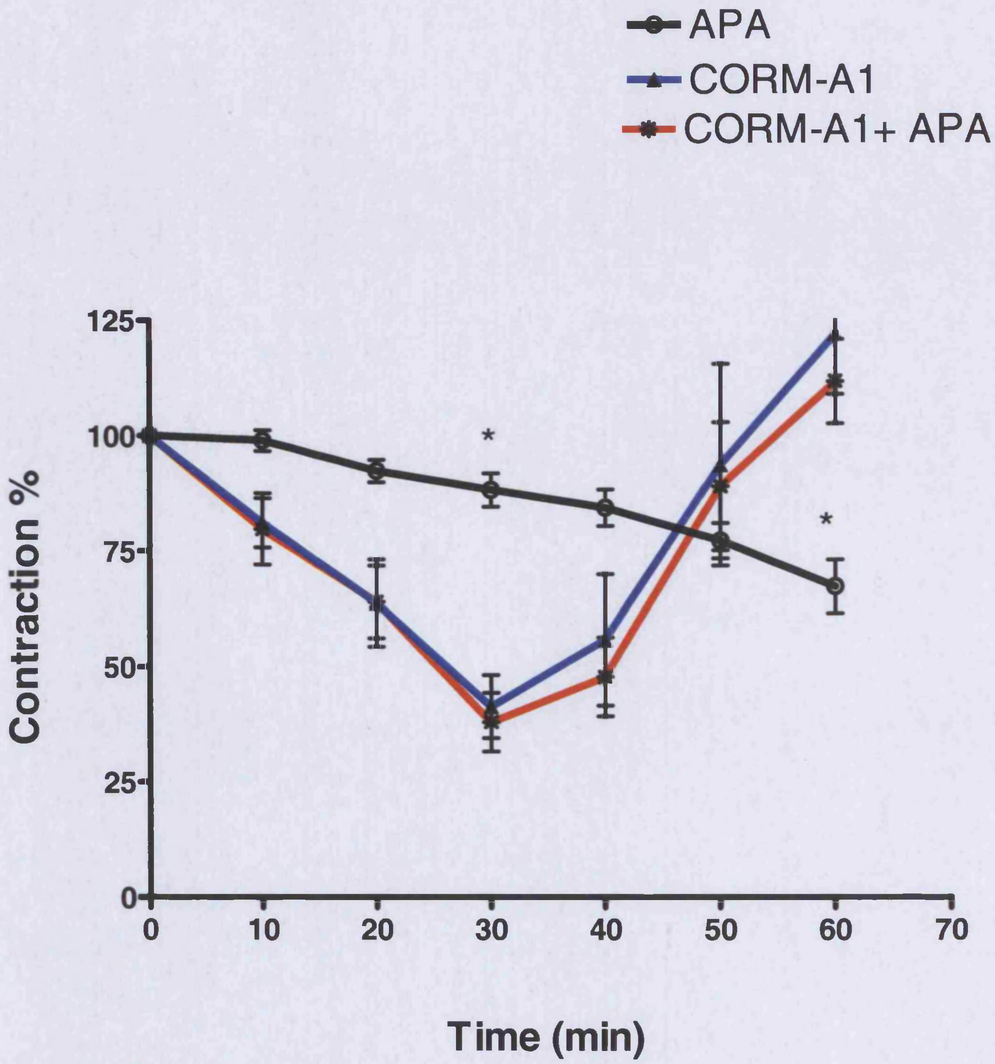


Figure 5.5B. SK_{Ca} channel blocker, apamine, did not affect CORM-A1-induced vasorelaxation.

Rings were incubated with 100 nM apamine (APA) 60 min prior to the addition of phenylephrine. APA did not affect CORM-A1mediated vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-A1 (80 μM) alone.

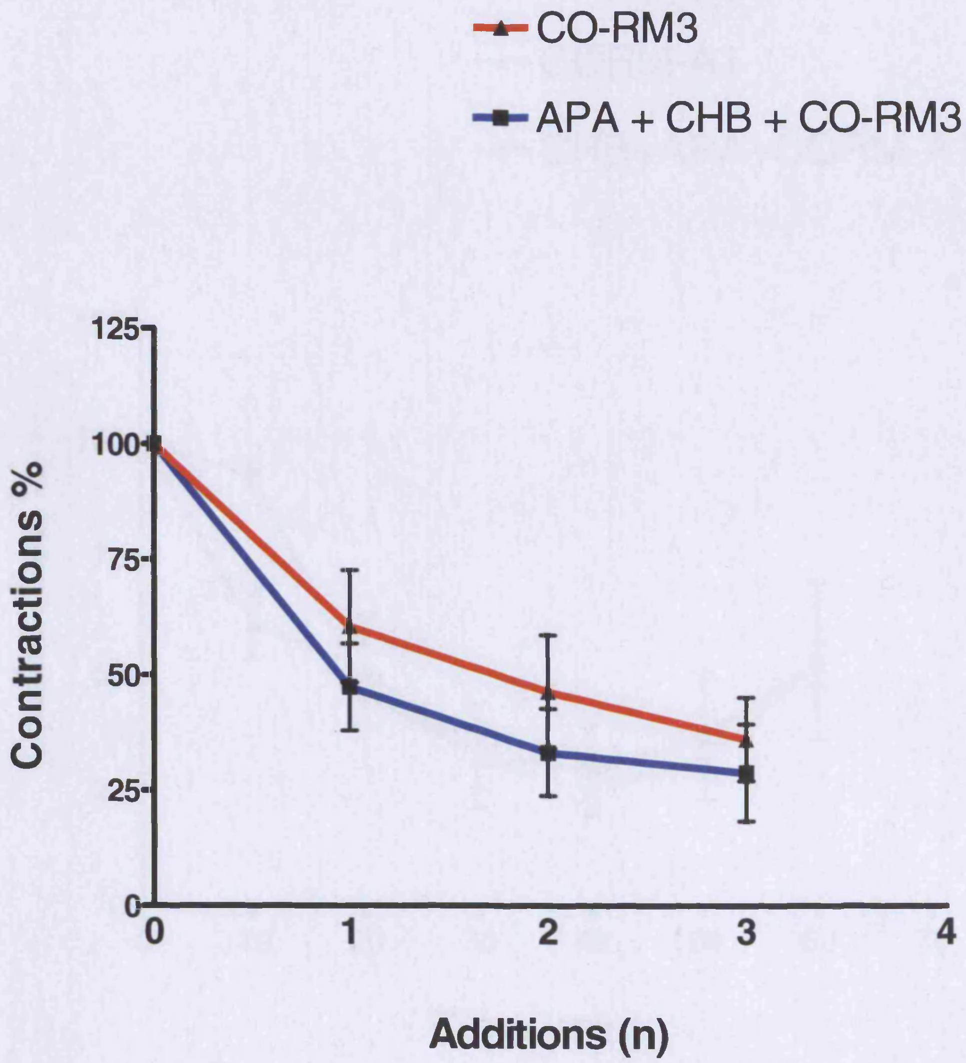


Figure 5.6A. Combination of SK_{Ca} and BK_{Ca} has insignificant effect on CORM-3-induced vasorelaxation.

Rings were incubated with both 100 nM apamine (APA) and 100 nM charybdotoxin (CHB) 60 min prior to addition of phenylephrine. Combination of APA and CHB had no significant effect on CORM-3-induced vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-3 (100 μM) alone.

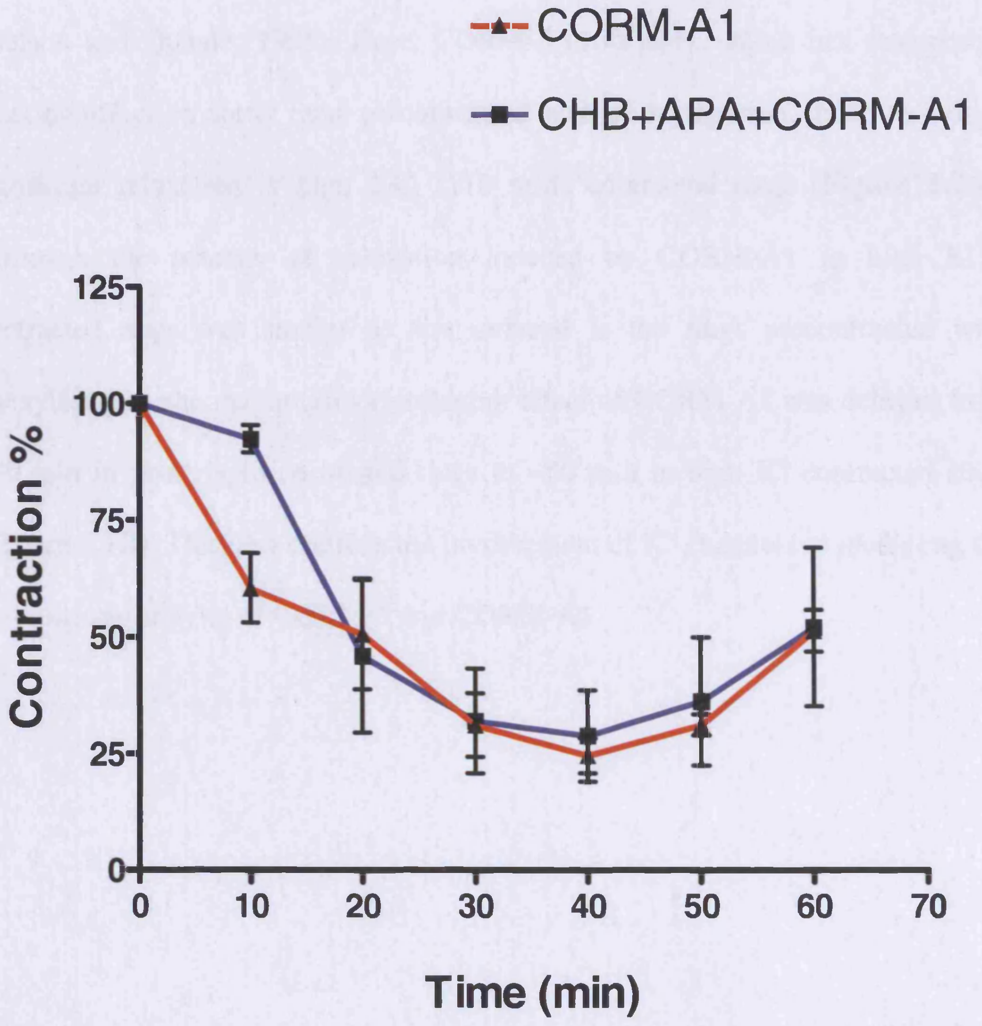


Figure 5.6B. Combination of SK_{Ca} and BK_{Ca} has insignificant effects on CORM-A1-induced vasorelaxation.

Rings were incubated with both 100 nM apamine (APA) and 100 nM charybdotoxin (CHB) 60 min prior to addition of Phe. Combination of APA and CHB has no effect on CORMA1-induced vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. $^{*}P<0.05$ compared to CORM-A1 (80 μM) alone.

5.2.5 *Effect of CO-RMs on high K^+-contracted rings*

At high external K^+, cell membrane potential and E_K are very close together thus preventing any membrane hyperpolarization and relaxation to K^+ channel openers (Nelson and Quayle, 1995). Here, CORM-3 (100 μM), which has remarkable relaxing effect on aortic rings precontracted with phenylephrine, failed to induce significant relaxation in high KCl (110 mM) contracted rings **(Figure 5.7A).** Although the potency of relaxation induced by CORM-A1 in high KCL contracted rings was similar to that induced in the rings precontracted with phenylephrine, the maximum vasorelaxing effect of CORM-A1 was delayed from ~30 min in phenylephrine treated rings to ~50 min in high K^+ contracted rings **(Figure 5.7B).** This data confirm the involvement of K^+ channels in mediating the vasorelaxing activity of CORM-3 and CORM-A1.

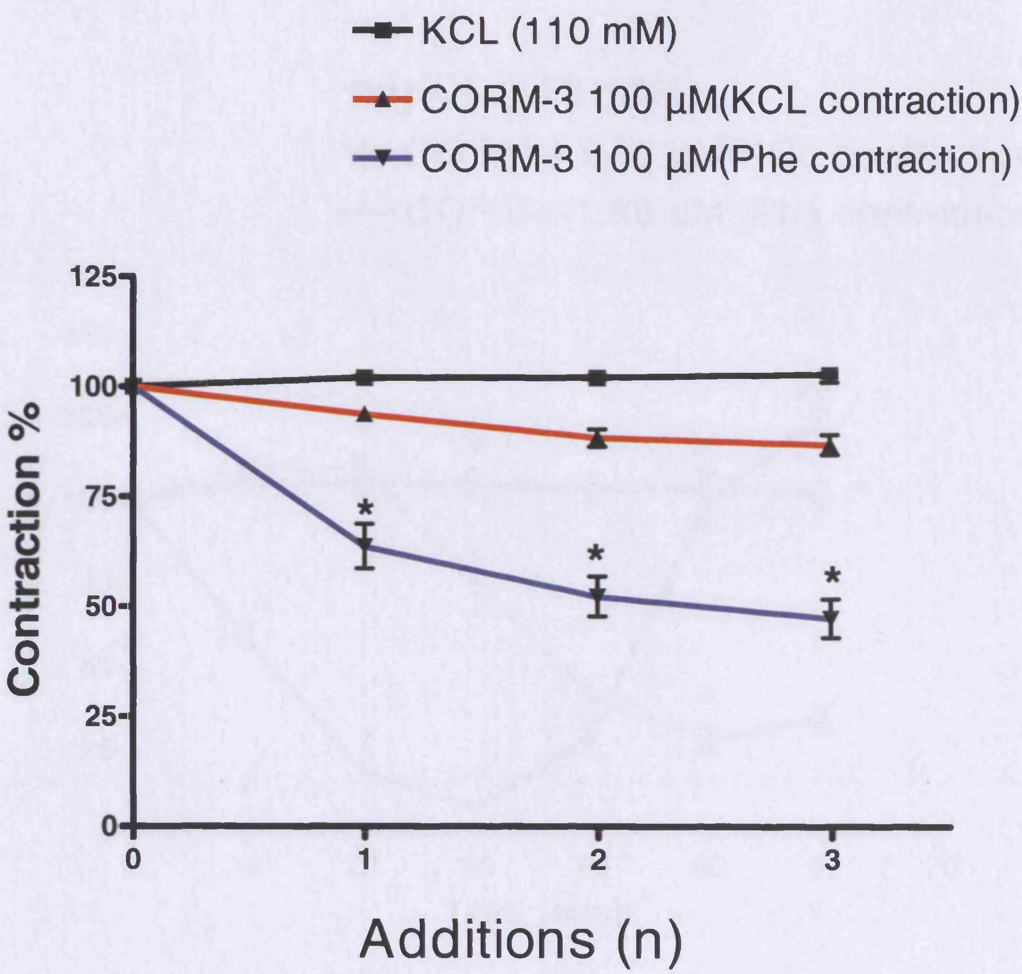


Figure 5.7A. CORM-3 has little effect on high KCl contracted rings.

Rings were precontracted with KCl (110 mM) prior to CORM-3 (100 μM) addition. CORM-3 failed to induce significant relaxation in KCL contracted rings. The vasorelaxing effect of CORM-3 (100 μM) on KCL precontracted rings were compared to the effect induced by same concentration of CORM-3 on Phe precontracted rings. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-3 (KCL contraction).

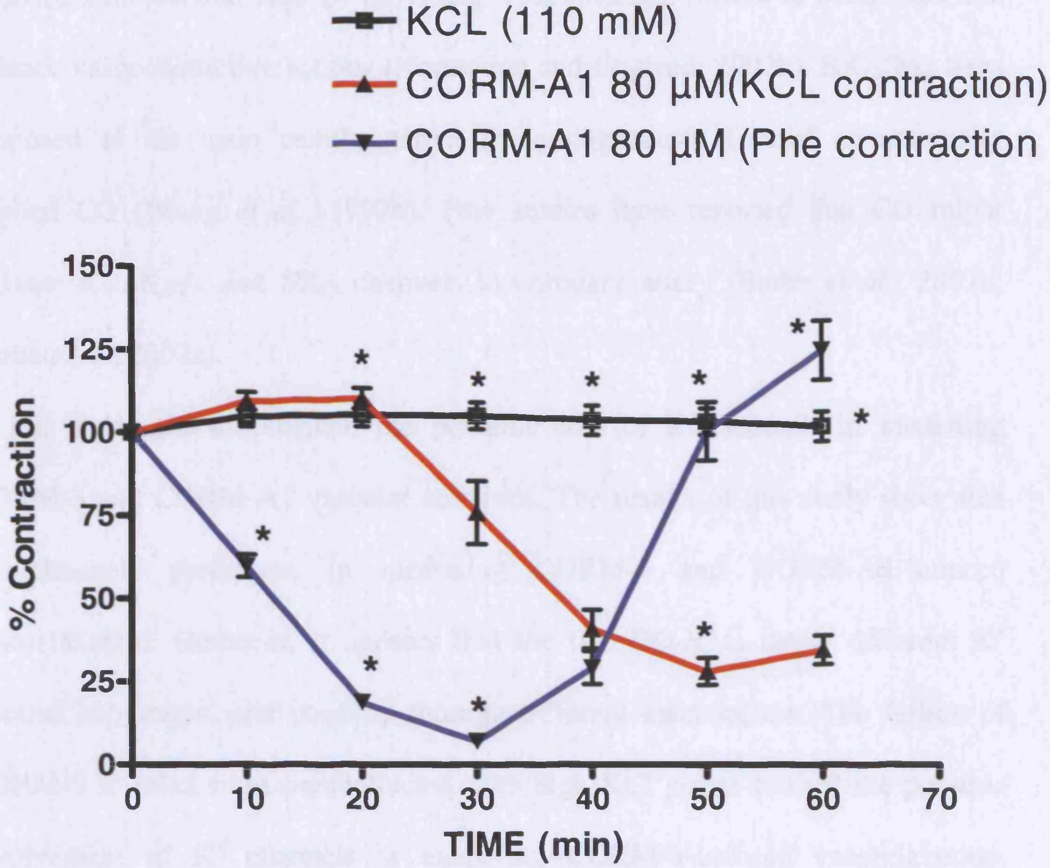


Figure 5.7B. High KCl delayed CORM-A1-induced vasorelaxation.

Rings were precontracted with KCl (110 mM) prior to CORM-A1 addition. CORM-A1 induced significant vasorelaxation in KCl pretreated rings but the maximum vasorelaxation was delayed to around 50 min after adding CORM-A1. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ±s.e.m. of 6-8 independent experiments. *P<0.05 compared to CORM-A1 (KCL contraction).

5.3 Discussion

K^+ channels have multiple functions in vascular tissues. Among these functions is regulation of vascular tone by providing a repolarizing current to counteract and balance vasoconstrictive actions (Korovkina and England, 2002b). BK_{Ca} has been proposed as the main cellular target for endogenously formed or externally applied CO (Wang *et al.*, 1997b). Few studies have reported that CO might activate K_V, K_{ATP}, and SK_{Ca} channels in coronary artery (Barbe *et al.*, 2002b; Barbe *et al.*, 2002c).

In this study we investigated the potential role of K^+ channels in mediating CORM-3 and CORM-A1 vascular activities. The results of this study show that K^+ channels participate in mediating CORM-3 and CORM-A1-induced vasorelaxation. However, it appears that the two CO-RMs target different K^+ channel subgroups, and possibly through different mechanisms. The failure of CORM-3 to relax rings precontracted with high KCl points toward the possible involvement of K^+ channels in mediating CORM-3-induced vasorelaxation. Furthermore, the fact that TEA, which is a nonslective inhibitor of various K^+ channels, completely reversed the CORM-3-induced vasorelaxation confirms the role of K^+ channels in mediating CORM-3-induced vasorelaxation. In the current results, the ability of TEA to moderately attenuate the vasorelaxing effect of CORMA1, also points toward the role of K^+ channels in mediating CORM-A1 vascular activities. Although high KCl did not prevent CORM-A1-induced vasorelaxation, it delayed its maximum vasorelaxation (from after 30 min in Phe precontracted rings to after 50 min in KCl precontracted rings) which indicate that K^+ channels might mediate the first part of CORM-A1-induced vasorelaxation.

The present results show that among different K^+ channel subgroups, K_{ATP}

channels play an important role in mediating the vasorelaxing properties of CORM-3. CO released from CORM-3 may activate soluble guanylate cyclase enzyme which increase intracellular cGMP. Elevation of intracellular cGMP either activates cGMP dependent protein kinase (PKG) or cross activates cAMP-dependant protein kinase (PKA) to stimulate K_{ATP} channels (Brayden *et al.,* 2002; Standen and Quayle, 1998). Although glibenclamide was the only specific K^+ channel subgroup blocker that attenuated CORM-3-induced vasorelaxation this finding does not completely exclude the roles of other K^+ channel subfamilies for two weighty reasons. Firstly glibenclamide only moderately attenuated CORM-3-induced vasorelaxation, whereas TEA and high KCl reversed it completely or significantly ($P< 0.05$) respectively. Secondly TEA as mentioned before is a nonselective blocker of K^+ channels which can at different concentrations (lower than those used in this study) induce partial block of other K^+ channel subtypes such as K_V and K_{Ca} channels (Nelson *et al.*, 1990). BK_{Ca} channels might be another mediator of CORM-3-induced vasorelaxation because charybdotoxin in the present results showed some inhibitory effect on the vasorelaxing activity of CORM-3 (it decreased the maximum vasorelaxation after third addition of CORM-3 from 70% to 52%) and this possibility is supported by a reported direct effect of CO on K_{Ca} in vascular SMCs (Wang *et al.*, 1997b). However, the inhibitory effect of charybdotoxin on CORM-3 was not statistically significant ($P< 0.05$). In addition, a combination of charybdotoxin and apamine potentiated, instead of attenuating, the CORM-3-induced vasorelaxation. Therefore the possible role of K_{Ca} in mediating CORM-3-induced vasorelaxation is undermined. The other possibility that can explain the gap between the complete and partial inhibition of TEA and glibenclamide respectively is that the different K^+ channels subgroups might interact comprehensively to mediate the vasorelaxing activities of CORM-3. This interaction might reflect the sophistication and diversity of K^+ channels.

The interaction between K^+ channels and CORM-A1 seems to be less complex as both TEA and 4-AP, elicited similar partial inhibition on CORM-A1-induced vasorelaxation and both TEA and high KCl delayed the maximum vasorelaxation induced by CORM-A1. These findings point toward the role of K_V channels in mediating the first part of CORM-A1-induced vasorelaxation. CO released by CORM-A1 might activate K_V channels directly or indirectly through elevation of intracellular cGMP level since cGMP has been suggested to partially participate in activating K_V channels (Satake and Shibata, 1997).

Our current results do not support other reported results which accentuate the role of K_{Ca} in mediating CO-induced vasorelaxation (Kodama *et al.*, 1997; Wang *et al.*, 1997a; Kodama *et al.*, 1997; Wang and Wu 2003; Xi *et al.*, 2004). There are some possible explanations for this difference between our results and those presented in other studies. Firstly, although the findings of other studies confirm the role of BK_{Ca} channels, they did not rule out the role of vascular K_V, K_{ATP} channels as targets for CO activity. Secondly, the expression of different K^+ channel subgroups is highly dependant on the tissue type and developmental age (Cao *et al.,* 2002; Nelson and Quayle, 1995; Tang and Wang, 2001; Archer *et al.,* 1996). Accordingly, the K_V and K_{ATP} not K_{Ca} channels might be the dominant subgroups in male adult Sprague Dawley rats examined in this study. Thirdly, the pattern of CO delivery to vascular tissue might explain the variation regarding the role of K^+ channel subtypes between our results and those published by other groups. Supporting this hypothesis it has been reported that the effect elicited by external CO was different from the endogenously produced CO (Hristov *et al.*, 2004; Naik and Walker, 2003). Additionally, the different patterns of CO release from CORM-3 and CORM-A1 (fast versus slow) might explain the difference in their cellular target. It seems that CO released rapidly from CORM-3 activates K_{ATP} channel subtypes whereas CO liberated slowly from CORM-A1 preferred K_V channels.

Taken together, the present data indicate that K^+ channels play a role in CORM-A1 and CORM-3-induced vasorelaxation mainly through activation of K_V and K_{ATP} channels respectively. It also shows that the kinetics of CO release from different CO-RMs might affect their pharmacological activities.

6 The Vascular Properties of Iron-Containing Carbon Monoxide-Releasing Molecules (CO-RMs)

In this chapter we continued our persistent search for molecules that can be safely used as carbon monoxide releasing molecules (CO-RMs) in biological systems. Therefore we have identified a new iron containing metal carbonyl molecule, here termed CORM-319 as another promising water soluble molecule that can release CO spontaneously in aqueous solutions and could be used safely for timed and measurable CO delivery into vascular tissue. Using an amperometric CO sensor, CORM-319 was found to have a fast pattern of CO release in physiological solutions (unpublished data from our group). Using aortic ring preparations we studied the vasorelaxing properties of CORM-319 and compared it to another three DMSO soluble CO-RMs. These CO-RMs, termed CORM-307, CORM-308 and CORM-314, are also iron containing metal carbonyls and have very similar chemical structures to CORM-319 as shown below.

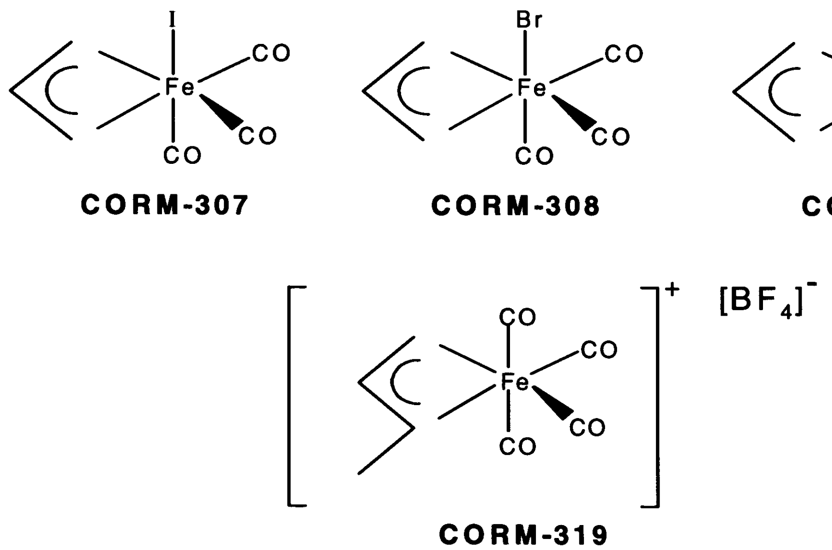


Figure 6.1A. Chemical structure of iron-containing CO-RMs (unpublished data from our group).

The only difference between these CO-RMs is the halide (I, Br or Cl) which coordinates in *trans* position to one of the CO groups bound to the metal center. In this project, using Alamar Blue technique, we also assessed the effect of iron containing CO-RMs on smooth muscle cell (SMCs) viability to see whether the difference in chemical structure has significant impact on their toxicity.

6.1 Materials and Methods

6.1.1 Preparation of solutions

CORM-307, 308, 314 and 319 were prepared as previously described Materials and Methods (Chapter 2). Stock solutions of CORM-319 (in distilled water) and CORM307, CORM-308 and CORM-314 (in DMSO) were prepared fresh and used within one min from preparation. The inactive form of CORM-319 (iCORM-319) was obtained by leaving CORM-319 in distilled water overnight at room temperature. The inactive forms of the other CO-RMs (iCO-RMs) were obtained by leaving them in DMSO for at least 48 h at room temperature. The iCO-RMs solutions were finally bubbled with a stream of nitrogen to remove any residual CO present in the solution. This inactivation process produced compounds that did not release CO. The solutions of iCO-RMs were tested with the myoglobin assay prior to experiments to verify its inability to liberate CO (unpublished data from our group).

6.1.2 Cell viability

Rat aortic smooth muscle cells (A7r5) were cultured as previously described in Materials and Methods (Chapter 2). Monolayers of A7r5 cells in 24 wells plates were divided into four groups. The first group was incubated with CORM-319 (10-500μM) and its inactive form iCORM-319 (10-500μM) for 24 h. The other three groups were treated for 24 h with increasing concentrations (10-100μM) of active and inactive forms of CORM-307, 308 and 314. Then the cell viability was determined in the SMCs using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK) as previously explained in Materials and Methods.

6.1.3 Aortic ring preparation

Transverse sections of aortic rings were prepared from adult Sprague-Dawley rats (300-400 g) and suspended under 2 g tension in oxygenated Krebs-Henseleit buffer as previously described in Materials and Methods (Chapter 2). The extent of vasorelaxation over one hour elicited by a single addition of CORM319 (12.5-100 μM) was monitored in aortic rings precontracted with phenylephrine (1 μM) and compared to the effect produced by iCORM-319 (100 μM). The vasorelaxing properties of a single addition of DMSO soluble CO-RMs (100 μM) was also assessed and compared to their inactive forms (iCO-RMs). The vasodilatory response was expressed as percentage of the vasoconstriction induced by phenylephrine.

6.1.4 Statistical analysis

Statistical analysis was performed using one-way and two-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $p<0.05$.

6.2 Results

6.2.1 Vasorelaxing properties of iron-containing CO-RMs

Pre-contracted aortic rings were treated with increasing concentrations of water soluble CORM-319 (12.5, 50 and 100 μM) and vasorelaxation was measured at different time points. CORM-319 induced fast and concentration-dependant vasorelaxation in phenylephrine contracted rings **(Figure 6.1).** The maximum vasorelaxation mediated by CORM-319 was reached after 10 min; this was quantified as 19.5+5.8 % at 12.5 μM, 36.4+6.8 % at 50 μM and 57.1+2.7 at 100 μM. On the other hand, iCORM-319 at 100 μM elicited only slight relaxation 8.8+6.2 % indicating that CO release from CORM-319 is the factor involved in the modulation of vascular tone. To a certain extent the vasorelaxing properties of the DMSO soluble CO-RMs were different. For instance, iCORM-307 at 100 μM produced very similar vasorelaxing pattern compared to its active form CORM-307 at the same concentration **(Figure 6.2)**. Both of them elicited gradual and progressive vasorelaxation that reached the maximum 60 min after adding the CO-RMs to the organ bath. Therefore, the vasorelaxation induced on the aortic rings cannot be contributed only to CO released from CORM-307. The vasorelaxing patterns of CORM-308 and CORM-314 were slightly different to that obtained with CORM-307. CORM-308 and 314 elicited gradual and progressive vasorelaxation which reached maximum after 60 min, whereas their inactive forms induced relatively weaker vasorelaxation which reached maximum after 10 min and remained more or less stable over the next 50 min **(Figure 6.3 and 6.4)**.

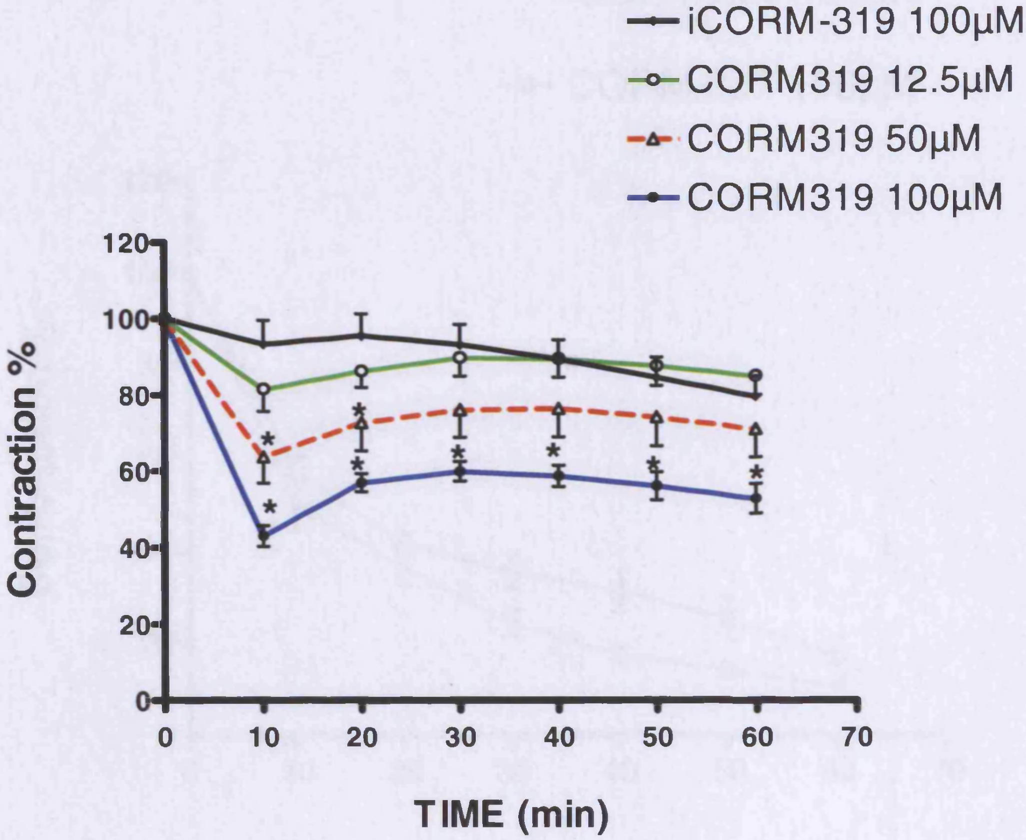


Figure 6.1. Effect of CORM-319 on vascular tone.

This graph shows the vasodilatory response of precontracted aortic rings to three different concentrations of CORM-319 (12.5, 50, and 100 μM). CORM-319 caused significant relaxation over time in a concentrated-dependent manner. Conversely, iCORM-319 the negative control elicited mild vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 5-6 independent experiments. *P<0.05 compared to iCORM-319 100 μM.

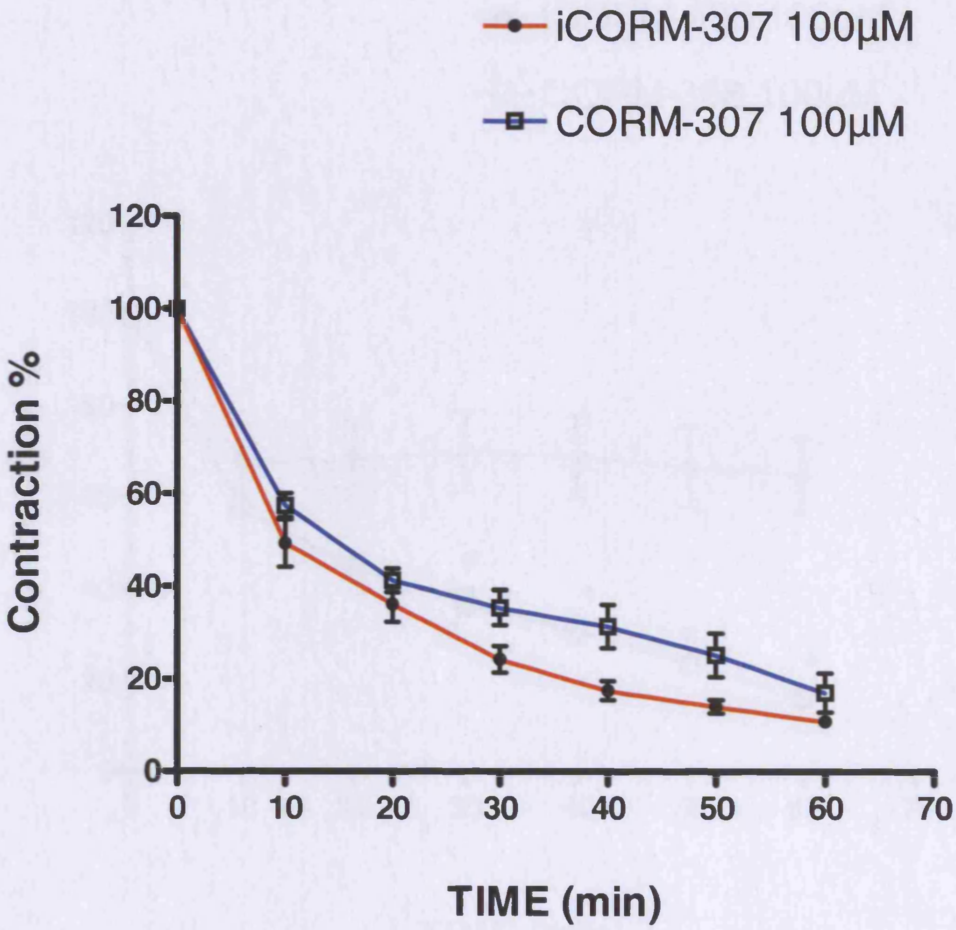


Figure 6.2. Effect of CORM-307 on vascular tone.

This graph shows the vasodilatory response of precontracted aortic rings to a single addition of CORM-307 (100 µM). CORM-307 caused significant relaxation over time in the contracted rings. iCORM-307 the negative control elicited similar pattern of vasorelaxation. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 5-6 independent experiments. *P<0.05 compared to iCORM-307 100 µM.

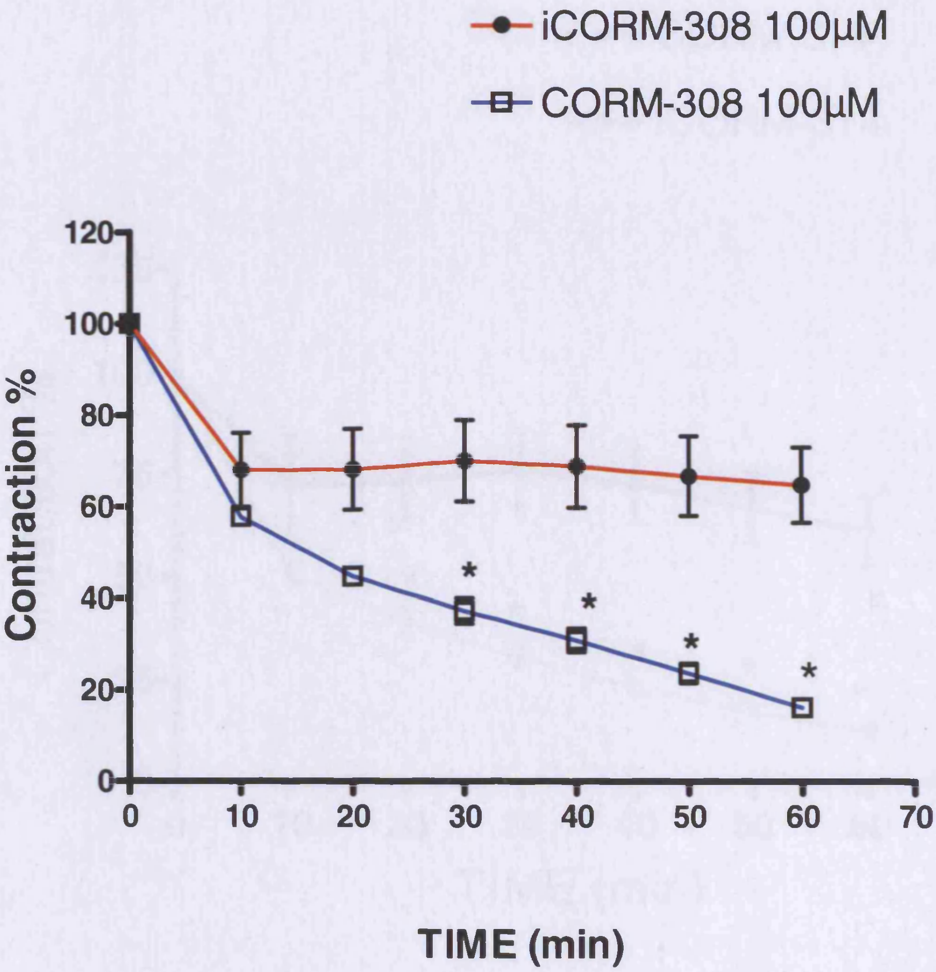


Figure 6.3. Effect of CORM-308 on vascular tone.

This graph shows the vasodilatory response of precontracted aortic rings to a single addition of CORM-308 (100 µM). CORM-308 caused significant relaxation over time in the contracted rings. iCORM-308 the negative control elicited weaker vasorelaxation pattern that remain stable over time. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 5-6 independent experiments. *P<0.05 compared to iCORM-308 100 µM.

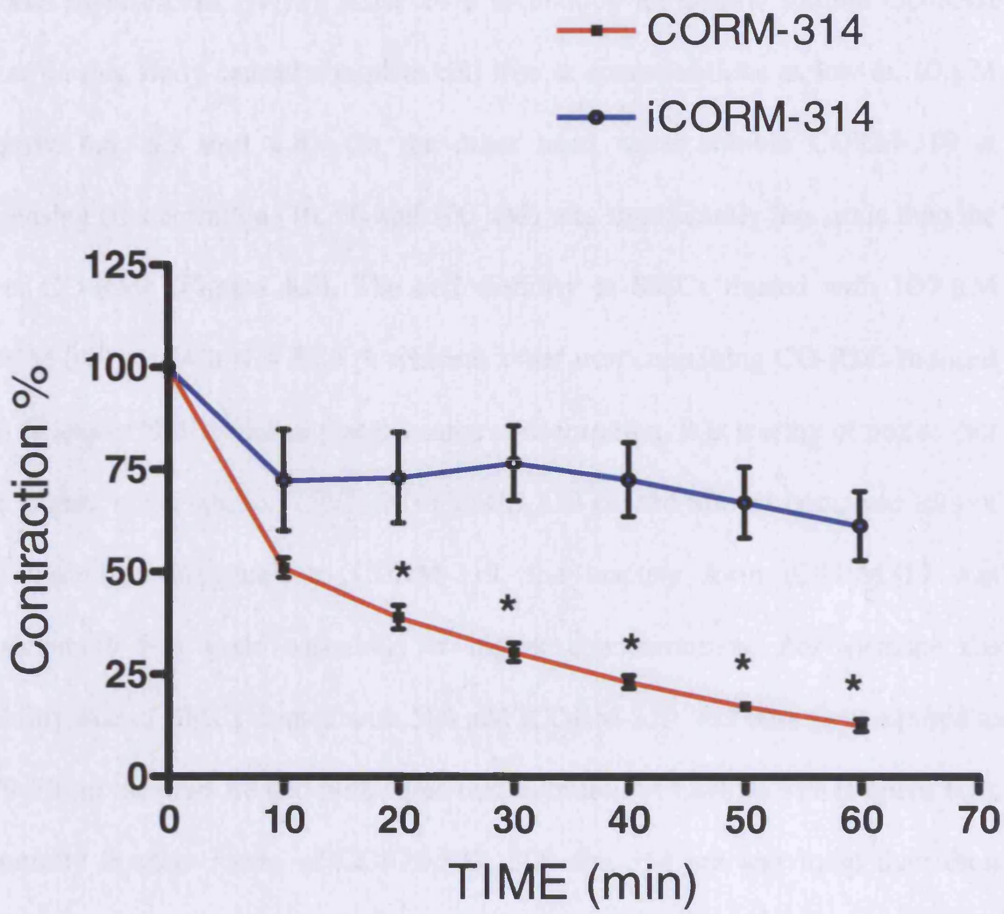


Figure 6.4. Effect of CORM-314 on vascular tone.

This graph shows the vasodilatory response of precontracted aortic rings to a single addition of CORM-314 (100 μM). CORM-314 caused significant progressive relaxation which reached maximum after 60 min. iCORM-314 the negative control elicited weaker vasorelaxation pattern compared to its active form. Vasodilatation is expressed as percentage of maximal precontraction. Data represent the mean ± s.e.m. of 5-6 independent experiments. *P<0.05 compared to iCORM-314 100 μM.

6.2.2 Effect of iron containing CO-RMs on SMCs viability

The effect of iron containing CO-RMs on cell viability was examined on rat aortic smooth muscle cells (A7r5). After 24 h incubation all DMSO soluble CO-RMs tested in this study caused complete cell loss at concentrations as low as 10 μM **(Figure 6.6, 6.7 and 6.8)**. On the other hand water soluble CORM-319 at increasing concentration (10, 50 and 100 μM) was significantly less toxic than the other CO-RMs **(Figure 6.5).** The cell viability of SMCs treated with 100 μM CORM-319 for 24 h was 58.8 % whereas other iron containing CO-RMs induced 100 % loss of SMCs viability at the same concentration. It is worthy of notice that at a higher concentration (500 μM) CORM-319 caused almost complete loss of cell viability. Compared to CORM-319, the inactive form iCORM319 was significantly less toxic especially at higher concentrations. For instance the viability rate of SMCs treated with 500 μM iCORM-319 was 60.8 % compared to 0.79 5% in the cells treated with same concentration of CORM-319 **(Figure 6.5).** Generally inactive forms of CORM-307, 308 and 314 are less toxic than their active forms especially at low concentrations (10- 50 μM). These findings point toward a role for released CO in mediating the toxic effect of iron containing CO-RMs.

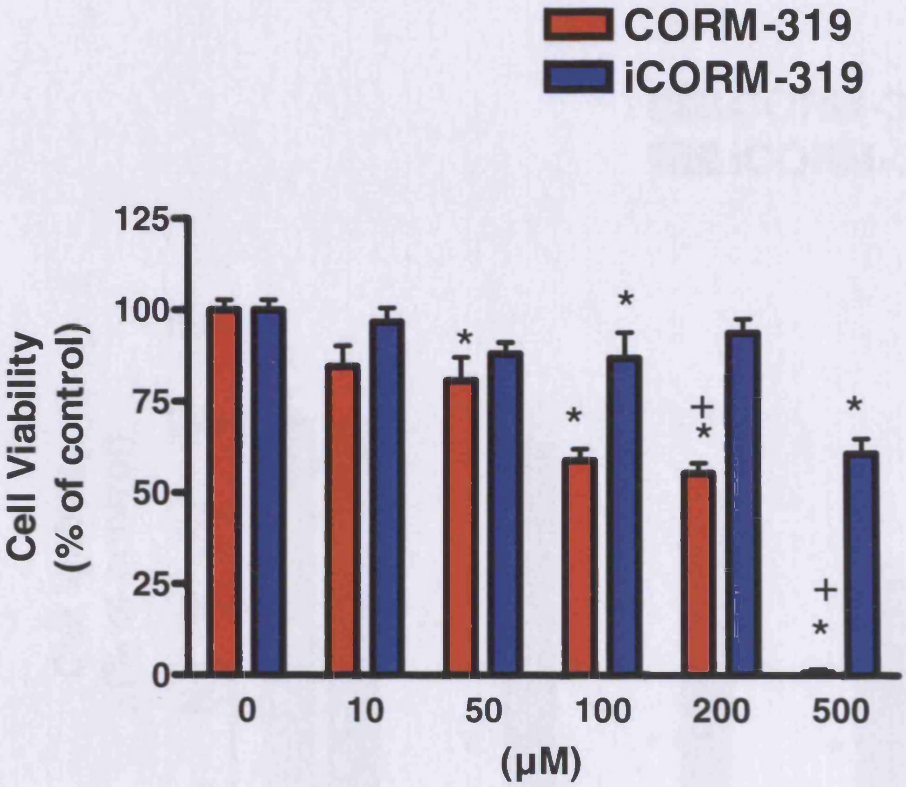


Figure 6.5. Effect of CORM-319 on aortic SMCs viability.

This graph compares the effect of CORM-319 (10-500 μM) and its inactive form iCORM-319 on A7r5 cell line viability. As shown in this graph the toxicity of CORM-319 increased with rise in concentrations and reached maximum at 500 μM. the inactive form iCORM-319 was relatively less toxic than CORM-319 especially at high concentrations. Data represent the mean ± s.e.m. of 4-6 independent experiments. $^{*}P<0.05$ compared to CORM-319 (0 μM). $^{+}P<0.05$ compared to related concentration of iCORM-319.

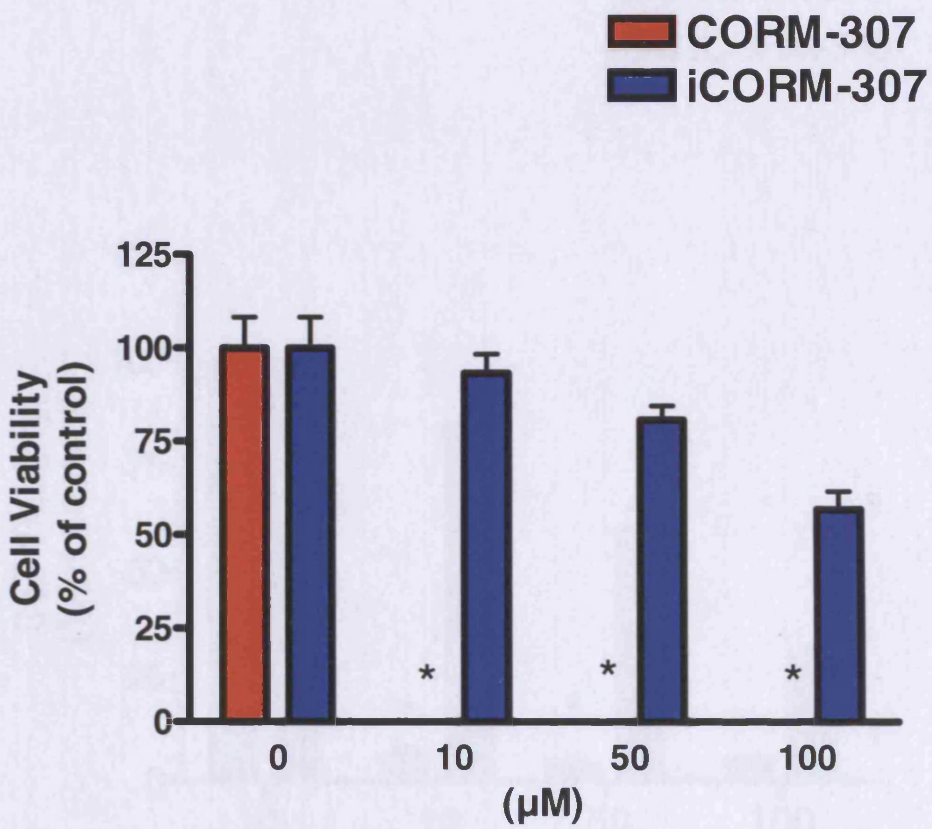


Figure 6.6. Effect of CORM-307 on aortic SMCs viability.

This graph compares the effect of CORM-307 and its inactive form iCORM-307 on A7r5 cell line viability. CORM-307 induced complete loss cell viability at 10 μM. iCORM-307 was less toxic than CORM-307 at all tested concentrations. Data represent the mean ± s.e.m. of 4-6 independent experiments. *P<0.05 compared to related concentration of iCORM-307.

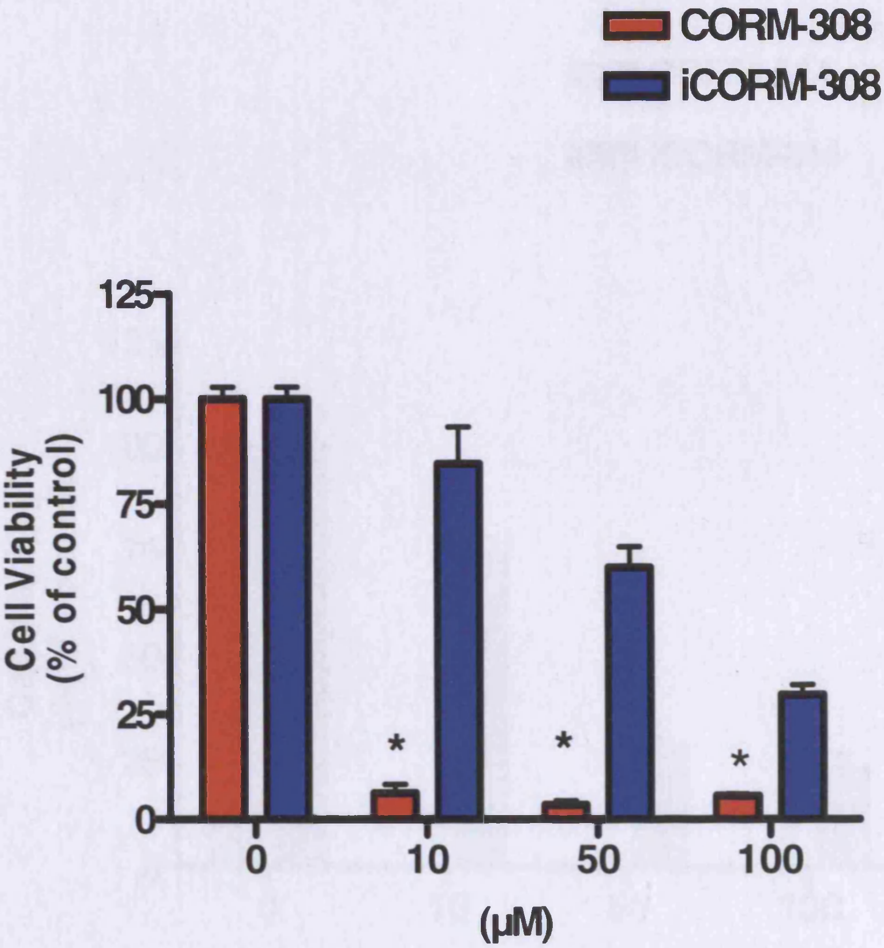


Figure 6.7. Effect of CORM-308 on aortic SMCs viability.

This graph compares the effect of CORM-308 and its inactive form iCORM-308 on A7r5 cell line viability. CORM-308 induced almost complete loss cell viability at 10 μM and its inactive form iCORM-308 had also significant toxic effect especially at high concentrations. Data represent the mean ± s.e.m. of 4-6 independent experiments. *P<0.05 compared to related concentration of iCORM-308.

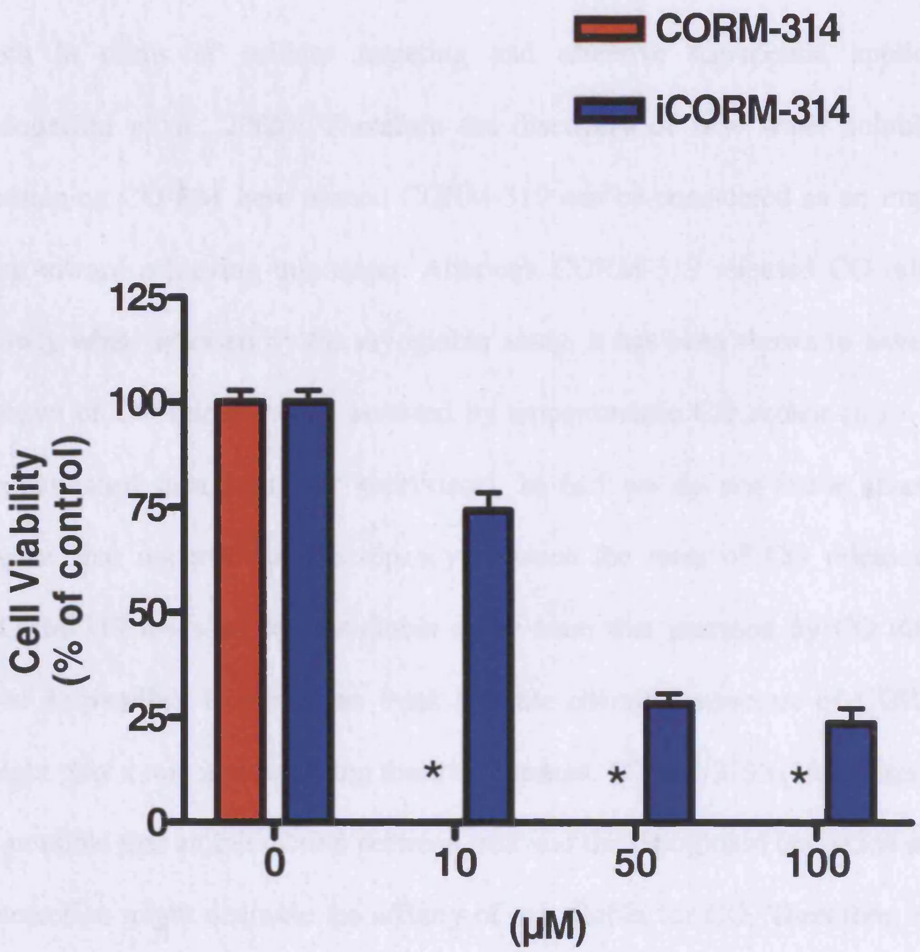


Figure 6.8. Effect of CORM-314 on aortic SMCs viability.

This graph compares the effect of CORM-314 and its inactive form iCORM-314 on A7r5 cell line. CORM-314 induced complete loss cell viability at 10 µM. iCORM-314 had also significant toxic effect especially at high concentrations. Data represent the mean ± s.e.m. of 4-6 independent experiments. *P<0.05 compared to related concentration of iCORM-314.

6.3 Discussion

The discovery of new CO-RMs with different chemical characteristics that allow the fine modulation of CO release has been proposed to offer more flexibility both in terms of cellular targeting and effective therapeutic applications (Motterlini *et al.*, 2005). Therefore the discovery of new water soluble iron containing CO-RM here termed CORM-319 can be considered as an important step toward achieving this target. Although CORM-319 released CO relatively slowly when assessed by the myoglobin assay, it has been shown to have a fast pattern of CO release when assessed by amperometric CO sensor ($t_{1/2}$~ 1 min) (unpublished data from our supervisor). In fact we do not know exactly the causes that underlie the discrepancy between the rates of CO released from CORM-319 assessed by myoglobin assay from that assessed by CO electrode (**see Appendix**). However we think that the chemical structure of CORM-319 might play a role in developing these differences. CORM-319 is iron based and it is possible that an interaction between iron and the myoglobin happened and this interaction might decrease the affinity of myoglobin for CO. Therefore, the rate of carbonmonoxyglobin formation was relatively slow compared to that obtained by CO electrode. On the other hand, CORM-3 hardly released any CO when assessed by CO electrode. Similarly the chemical structure of CORM-3 might underlie this discrepancy. We think that the ruthenium backbone of CORM-3 might precipitate on the CO electrode and decrease the available space for CO reading. Thus the rate of CO release assessed by CO electrode was not comparable to that evaluated by the myoglobin assay. Interestingly, CORM-A1, a very pure salt that does not contain iron or heavy metal, had similar patterns of

CO release when assessed by either myoglobin assay or CO electrode. Based on these findings, it is sensible to conclude that the chemical structure of a given CO-RM should be considered in interpreting data from different CO measuring techniques. Therefore we think the CO electrode assay is the best available assay to assess the rate of CO release from CORM-319, whereas myoglobin assay is the appropriate assay to evaluate the rate of CO liberated from CORM-3. For CORM-A1, a pure salt, both assays will have similar results. Thus the fast pattern of CO release detected by amperometric CO sensor might explain the vasorelaxing properties of CORM-319 on precontracted aortic rings. At increasing concentrations (12.5, 50 and 100 μM) CORM-319 elicited fast vasorelaxation that reached a maximum after 10 min. Since 100 μM iCORM-319 elicited only slight and significantly less relaxation these data indicate that CO released from CORM-319 is responsible for the observed effect.

Although DMSO soluble CORM-307 induced significant progressive relaxation in contracted aortic rings, this vasorelaxation cannot be attributed only to the released CO as the inactive form iCORM-307 elicited a similar pattern of relaxation. The other two DMSO soluble molecules CORM308 and CORM-314 elicited gradual and progressive significant vasorelaxation and their inactive forms also induced vasorelaxation but it was milder than that elicited by their active forms. The difference in vasorelaxing scale between the active and inactive forms might point toward a partial role played by CO released from CORM-308 and 314 in augmenting the vasorelaxing properties of these molecules. However, the vasorelaxation induced in contracted rings might be due to damage of aortic SMCs as both CORM-308 and 314 caused a complete loss of SMCs viability at concentrations as low as 10 μM.

The current SMCs viability results revealed that water soluble CORM-319 is the least toxic molecule among all the iron containing CO-RMs examined in this project. Using the Alamar Blue technique, all DMSO soluble CO-RMs induced complete loss of SMCs viability at 10 μM concentration, whereas 84.5 % of the cells treated with the same concentration of CORM-319 remained alive after 24 h. However, the toxicity of CORM-319 increased with rising concentrations. For example, CORM-319 induced only 41.2 % decrease in cell viability at 100 μM but at 500 μM it induced almost complete loss of cell viability. Another interesting finding in our results is that iCORM-319 was significantly less toxic than its active form especially at high concentrations (100-500 μM). This finding suggests that CO released from high concentrations of CORM319 have potential toxic effects on aortic SMCs. Similarly it seems that the CO bound to other iron containing CO-RMs plays an important role in boosting their cytotoxicity. Bearing in mind that one mol of CORM-319 releases one mol of CO, which is the same amount released by one mol from any other water insoluble CO-RMs, it seems that the toxicity of a released CO gas can be significantly affected by the chemical structure of the CO releasing molecule. That might explains the low toxic effect exerted by CO released from 10 μM CORM-319 compared to same concentration of CORM-307. Additionally it might explain the different effects on SMC imposed by CORM-3 and CORM-319, which are both water soluble and appear to have similar patterns of CO release.

In summary, our current results show that CORM-319 which is a new water soluble iron containing metal carbonyl is a promising compound that can spontaneously deliver CO into vascular tissues. Compared to other water insoluble iron containing CO-RMs tested in this study, CORM-319 elicits concentration-dependant vasorelaxation at concentrations that are relatively safe to aortic SMCs. Our data suggest that CO released from this iron carbonyl complex is responsible for the observed vasorelaxation. It also suggests that CO released from CORM-319 at high concentrations (100-500 μM) might have negative effects on aortic SMCs viability.

Furthermore our current data reveal that the small difference in the chemical structure between CORM-319 and the other iron based CO-RMs tested in this project has a great impact on their vascular activities.

7 General Discussion

7.1 Assessment of Methodology

An *ex vivo* model of aortic ring preparations was used in this project to assess the vasorelaxing properties of CO-RMs. In fact isolated vessel preparations have been extensively used to examine changes in contractile tension in blood vessels in response to various vasoconstricting and vasodilating compound (Jones *et al.,* 1986; Karaki *et al.,* 1987; Calderone *et al.*, 1996). Many advantages tempted us to use this model. Firstly, because the blood vessels are examined *in vitro*, the environment of the vessels can be accurately regulated, whereas many parameters can affect the result of *in vivo* studies (Jones *et al.,* 1986; Karaki *et al.,* 1987). Secondly, the concentration of drugs that are added to the bathing medium to which the vessels is exposed can be accurately established. Thirdly, in contrast to isolated aortic zig-zag or helical strip preparations, the isolated ring technique usually conserves the endothelial layer (Pual *et al.,* 1988; Calderone *et al.*, 1996). Fourthly, with proper training, the ring preparation is simple to prepare and to mount on hooks for recording tension. However, convincing criticism has also been directed against this *ex vivo* model. Firstly, it may be difficult to assess the effect of drugs working on presynaptic mechanisms using this preparation (Karaki *et al.,* 1987). Therefore, the effect of adrenergic compounds with potential vasodilating effects can not be assessed using this technique (Karaki *et al.,* 1987). Secondly, the response of isolated blood vessels towards some agent varies according to the type of blood vessel and species examined (Jones *et al.,* 1986; Karaki *et al.,* 1987; Calderone *et al.*, 1996). These variations can be attributed to the complexity of vascular SMC architecture. Thirdly, though this model has been widely employed, there is no unanimous consensus that the physiology of isolated blood vessels mimics that of *in vivo* vessels (Jones *et al.,* 1986). However, despites these disadvantages, isolated blood vessel models have provided us with very useful results; and in most cases the response of isolated vessels qualitatively reflected the response of the blood vessels *in vivo* (Jones *et al.,* 1986).

In the current project three consecutive additions of CORM-3 were used to assess the vasoactive properties of CORM-3 because the vasorelaxing pattern induced by CORM-3 usually reached a plateau after 6-8 min. Therefore, second and third additions were added to have an accumulative effect of CORM-3. The rings were monitored for 8 min after the last addition. On the other hand, only one addition of CORM-A1 was used to assess the vasoactivity of CORM-A1 because one addition of CORM-A1 was enough to induce almost complete reversal of contraction induced by phenylephrine, therefore there was no need to make extra additions of CORM-A1. It is worth notice that CORM-A1 was introduced to our laboratory around 6 months after CORM-3. The recontraction of aortic rings that follow the vasorelaxation induced by CORM-A1 prompted us to monitor the effect of CORM-A1 for one hour until the recontraction reach a plateau. Similarly we ran a group of experiments **(Figure 3.2)** to assess the response of precontracted aortic rings to one addition of CORM-3 (100 μM) over one hour.

The vasorelaxing effect of exogenous CO was not assessed in the present study because in our opinion it is difficult to precisely control the amount of CO gas that is added to biological preparations. Many studies, analysed in Introduction (Chapter 1), have demonstrated the vasorelaxing effect of exogenous CO gas in different vascular beds. However, there has been a lack of agreement among scientist about the real concentrations of CO gas in physiological buffers. Many studies have reported the discrepancy between the calculated concentrations and real concentrations measured in experimental buffers. For example in a study conducted on cerebral arteries it was demonstrated that the measurable concentrations of CO in their experimental models were around 99% less than the expected concentrations (Komuro *et al.,* 2001). In another study the concentration of CO in the buffer was 50% less than the theoretical one (Marks *et al.,* 2002a). Therefore we thought that use of external CO gas would complicate instead of clarifying our results.

In the present study, the Alamar Blue technique was used to assess aortic SMCs viability *in vitro*. The one step Alamar Blue assay, which is an indicator of mitochondrial metabolic activity, particularly of the metabolic chain reaction, has gained popularity as a simple technique to assess the cytotoxiciy of different compounds (Nakayama *et al.*, 1997; O'Brien *et al.*, 2000; Miret *et al.,* 2005). It is very simple, extremely stable, fast, and least toxic to the cells, accurate and sensitive assay providing certain precaution are taken (Nakayama *et al.*, 1997; O'Brien *et al.*, 2000; Miret *et al.*, 2005). However, the density of cells examined, Alamar Blue dye concentration, incubation time, and the interaction between the dye and the compound to be used, can all affect the result of the assay (O'Brien *et al.*, 2000; Nakayama *et al.*, 1997). In fact more sensitive assays to evaluate cell viability through different pathways have now been introduced, and combinations of different assays for different aspects of cell death has been proposed as the best way to assess the cytotoxicity of a given compound (Miret *et al.*, 2005). However, despite its drawbacks, Alarm Blue assay remains a useful indicator for drug toxicity (O'Brien *et al.*, 2000).

7.2 Assessment of the Pharmacological Tools Used in This Study

One major problem in pharmacological research is the insufficient specificity of the tools of the trade (Cao *et al.,* 2002). The selective inhibitors used in this study to assess the cellular pathways targeted by CORM-3 and CORM-A1 are no exception. ODQ, (IH-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one), was identified by Garthwaite and colleagues, around ten years ago, and considered as first potent and selective inhibitor of soluble guanylate cyclase, that does not affect either NO nor NO synthase (NOS) activity (Garthwaite *et al.*, 1995). Since that time, ODQ has been extensively used as the most selective sGC inhibitor (Cao *et al.,* 2002). However, Feelisch and colleagues have shown that ODQ lacks specificity for sGC and interferes with other heme proteins such as cytochrome P-450 and NOS (Feelisch *et al.*, 1999). Therefore, the authors suggested that particular caution should be taken when assessing any vasodilators that might be affected by NOS activity (Feelisch *et al.*, 1999). Bearing in mind that in our studies vasorelaxation of aortic rings by 100 μM CORM-3 was completely inhibited by L-NAME, the NOS inhibitor, and ODQ significantly inhibited the vasorelaxation induced by the same concentration of CORM-3, an important question emerged; was the inhibitory effect of ODQ on CORM-3 due to cross inhibition of NOS activity? In order to rule out this possibility and support the role of cGMP in mediating the CORM-3-induced vasorelaxation we tested the direct effect of CORM-3 on cGMP production in aortic tissue extracts. CORM-3 significantly increased the cGMP production, especially after first and second additions, which correlates with the maximum inhibitory effect of ODQ on the vasorelaxation of aortic rings after first and second additions of CORM-3. Therefore these results confirm the role of the sGC/cGMP pathway in mediating vasorelaxation induced by CORM-3. As our results showed that CORM-A1 action was independent of endothelium or NOS activity, ODQ was considered an appropriate pharmacological tool to confirm the involvement of sGC/cGMP pathway in mediating the vasorelaxing effect of CORM-A1.

YC-1 is another pharmacological tool used in this project to verify the contribution of sGC/cGMP to CORM-3 or CORM-A1 vascular activity. Several reports have described YC-1 as directly activating sGC in vascular SMC and platelets (Ko *et al.*, 1994; Friebe *et al.*, 1996; Mulsch *et al.*, 1997). It has been widely used as a pharmacological tool to activate sGC/cGMP pathway in cultured cells or isolated tissues (Chun *et al.*, 2004). However, YC-1 was shown to have cGMP-independent inhibitory effects on K_V and K_{Ca} channels in cultured pituitary gland cells (Wu *et al.*, 2000). These results have been expected to confuse the interpretation of studies which use YC-1 specifically as an activator of sGC (Wu *et al.*, 2000). This expectation does not seem to apply to our results for two reasons: 1) inhibition of K_V and K_{Ca} channels depolarizes the vascular wall and induces vasoconstriction, which was not the case in our system, where incubating the aortic rings with YC-1 attenuated the vasoconstriction induced by phenylephrine; 2) YC-1 potentiated the vasorelaxing properties of CORM-3 and CORM-A1. Therefore, it is sensible to conclude that the synergistic effect of YC-1 with CORM-3 and CORM-A1 was mainly due to activation of sGC/cGMP system not to inhibition of K_V and K_{Ca} channels. Taken together, we believe we can safely say that ODQ and YC-1 were the proper pharmacological tools to examine the involvement of sGC/cGMP in mediating the vasorelaxing properties of CORM-3 and CORM-A1.

Potassium (K^+) channels are perhaps more sophisticated than any other ionic channels in vascular SMCs (Cao *et al.*, 2002). K^+ channel inhibitors have provided us with invaluable information about the properties and functions of K^+

channels (Olesen *et al.*, 1994; Nelson and Quayle, 1995).

Charybdotoxin (ChTX), a minor component of scorpion venom, reversibly blocks big conductance calcium-activated K^+ channels (BK_{Ca}) (Miller *et al.*, 1985). Charybdotoxin and other K_{Ca} inhibitors successors have provided us with unique tools not only to investigate the functional role of K^+ channels but also to develop the molecular pharmacology of these channels (Garcia *et al.*, 1995). Although it has been shown to block some other K^+ channels in other tissues, charybdotoxin appears to be selective for the K_{Ca} channels in arterial SMCs (Nelson and Quayle, 1995). Charybdotoxin was also reported to inhibit intermediate conductance K_{Ca} channels (IK_{Ca}) (Nelson and Quayle, 1995). However, IK_{Ca} do not seem to be involved in SM contractile functions. Instead it was suggested to be involved in non-excitable functions such as regulation of SMC growth (Neylon *et al.*, 1999). Therefore, we think that charybdotoxin was a proper choice to investigate the involvement of BK_{Ca} in conducting the vasorelaxing properties of CORM-3 and CORM-A1.

The functional role of small conductance K_{Ca} Channel (SK_{Ca}) was also assessed in this study. Three different subtypes of SK_{Ca} have been cloned and identified, SK1, SK2, and SK3 (Vergara *et al.*, 1998; Coghlan *et al.*, 2001; Liegeois *et al.*, 2003). In fact SK_{Ca} is classified pharmacologically according to their sensitivity to venom toxin apamin, into apamin sensitive and apamin insensitive SK_{Ca} channels (Vergara *et al.*, 1998; Bond *et al.*, 1999; Coghlan *et al.*, 2001). SK2 and SK3 are apamin sensitive, whereas SK1 seems to be apamin insensitive (Kohler *et al.*, 1996; Vergara *et al.*, 1998; Coghlan *et al.*, 2001). The localization and function of SK1 in vascular tissue is not clear. However, apamin alone or in combination with charybdotoxin, has been reported to inhibit arterial relaxations to various vasodilators, such as acetylcholine, and L-citrulline in numerous vascular beds (Ruiz and Tejerina 1998; Qiu and Quilley 2001). Therefore, in the light of available information regarding the SK_{Ca} channels, apamin remains a reasonable choice to study the possible role for SK_{Ca} in mediating the vasorelaxing properties of the CORM-3 and CORM-A1.

Voltage-dependant potassium channels (K_V), widely expressed in vascular tissue, are the most sophisticated K^+ channels subgroup (Cao *et al.,* 2002; Korovkina and England, 2002b). 4-aminopyridine (4-AP) is considered the most selective known blocker of K_V channels in vascular smooth muscle cells (Nelson and Quayle, 1995). Therefore, 4-AP has been used to differentiate K_V currents from K_{Ca} currents, which are also stimulated by membrane depolarization (Nelson and Quayle, 1995). 4-AP sensitive K_V channels have been identified in all vascular smooth muscle tissues investigated to date (Cole *et al.,* 1996). However 4-AP insensitive K_V channels have been identified in some vascular SMCs (Cao *et al.,* 2002). In rat aortic artery K_V2.1 channel, which is K_V channel subtype sensitive to millimolar concentrations of 4-AP, was reported to play an important role in the regulation of contractility of rat aorta (Tammaro *et al.*, 2004). Hence, we think that 4-AP was essential to assess the involvement of K_V channels in vasorelaxation induced by the tested CO-RMs. One of the pitfalls of 4-AP is that it might cross inhibit ATP dependant K^+ channels (K_{ATP}) in vascular tissue (Nelson and Quayle, 1995). However, the failure of 4-AP to affect the vasorelaxing properties of CORM-3, which was attenuated by K_{ATP} channel inhibition, points toward the absence of a significant inhibitory role of the 4-AP on K_{ATP} in our system.

Arterial K_{ATP} channels, the last K^+ channel subgroup to be examined in this study, are important regulators of vascular tone (Sampson *et al.*, 2004). They form a focal point for signalling by many modulators of vascular smooth muscle tone (Sampson *et al.*, 2004). The K_{ATP} channel is a hetero-octamer comprising two subunits: the pore-forming subunit Kir6.x (Kir6.1 or Kir6.2) and the regulatory subunit sulfonylurea receptor SUR (SUR1, SUR2A, or SUR2B) (Seino *et al.*, 1999; Cao *et al.*, 2002; Zhuo *et al.*, 2005). K_{ATP} channels are inhibited by glibenclamide, the oral hypoglycaemic sulfonylurea drug (Nelson and Quayle, 1995; Quayle *et al.*, 1997b; Sobey *et al.*, 2001; Jackson *et al.*, 2005). Glibenclamide appears to be selective for K_{ATP} channels and does not block other ion channels (Nelson and Quayle, 1995). However, the affinities of the SUR1, SUR2A, and SUR2B to glibenclamide are different (Cao *et al.*, 2002; Zhuo *et al.*, 2005). It was reported that SUR1 subunit binds glibenclamide with a dissociation constant (K_d) around 1 nM, while the K_d of SUR2A subunit is 1.2 μM (Cao *et al.*, 2002). Therefore, the concentration of glibenclamide used in this study (10 μM), would inhibit all SUR subunits.

Tetraethylammonium (TEA) is a non selective K^+ channel blocker used to probe structurs and functions of K^+ channels (Sansom *et al.*, 2002; Korn and Trapani 2005). The TEA can induce a half block of K_{Ca}, K_{ATP}, and K_V channels at concentrations ranged from 0.2 to 10 mM (Nelson and Quayle, 1995; Cao *et al.*, 2002). Therefore, the TEA concentration (30 mM) used in this study would inhibit all the above mentioned K channels subtypes.

In the current study, TEA completely reversed the CORM-3-induced vasorelaxation of aortic rings, whereas glibenclamide partially reversed it, and the other K^+ channel subtypes inhibitors had no significant effect on CORM-3. There are three possibilities for this discrepancy between the effect of TEA and glibenclamide. Firstly, the presence of unidentified TEA sensitive K^+ channel subtypes in aortic SMCs that are not sensitive to the inhibitors used in this study. Secondly, the presence of other ion channels or cellular pathways that is sensitive to TEA. Thirdly, K_V or K_{Ca} channels might provide alternative pathways to CORM-3 when K_{ATP} is blocked. Therefore, once TEA blocked K_{ATP}, K_V, and K_{CA} channels simultaneously, the vasorelaxing effect of CORM-3 was completely abolished. On the other hand the interaction between CORM-A1 and K^+ channels was less complex, as both TEA and 4-AP induced similar partial inhibition of CORM-A1- induced vasorelaxation. This suggests the K_V to be the only K^+ channel subtype targeted by CORM-A1.

Taken together the current results showed that both sGC and K_{ATP} channels are involved in mediating the vasorelaxing properties of CORM-3, whereas sGC and K_V channels are involved in that of CORM-A1. The question is how CO-RMs can interact with these cellular pathways to induce their actions?

CORM-3, through its released CO, might bind the haem moiety of sGC enzyme to induce a four to six fold increase in its activity (Stone and Marletta 1994; Kharitonov *et al.*, 1995). sGC catalyzes the conversion of GTP to cGMP which in turn stimulates a cascade of reactions ended by smooth muscle cell relaxation (Stone and Marletta 1994; Kharitonov *et al.*, 1995; Sharma and Magde 1999). CORM-3 can modify K_{ATP} channels either directly or indirectly. Like many other identified K_{ATP} channel openers, CORM-3 might interact with the SUR subunit of K_{ATP} channels (Lawson *et al.*, 2000; Coghlan *et al.*, 2001; Mannhold *et al.*, 2004; Moreau *et al.*, 2005). Indeed, SUR within the channel complex provides a major pharmaceutical target as it bind many drugs, K_{ATP} channel openers and blockers, capable of up-or down- regulating channel activity (Lawson *et al.*, 2000;

Mannhold *et al.,* 2004; Moreau *et al.*, 2005). Therefore, CORM-3 might bind directly to SUR subunit to activate the channel. Alternatively, CORM-3 might indirectly modify K_{ATP} channels through increased cGMP production which is associated with increase in K_{ATP} channels activity (Kubo *et al.*, 1994; Miyoshi *et al.,* 1994; Murphy and Brayden 1995). cGMP, through the subsequent activation of protein kinase G (PKG), can influence K_{ATP} channels activity directly, or indirectly through cross-activation of protein kinase A (PKA) (Quayle *et al.,* 1997a; Standen and Quayle, 1998).

In contrast to K_{ATP} channels which have been the most widely explored of the K^+ channels in terms of their therapeutic potential, there have been relatively few reports of the modulation of K_V channels by vasodilator agents in systemic vascular smooth muscle (Standen and Quayle 1998; Coghlan *et al.*, 2001). The cGMP stimulation has been associated with acetylcholine induced K_V channels activation (Satake *et al.*, 1997). In addition it was also reported that phosphorylation by PKA, enhance the K_V channel current in vascular smooth muscle cells (Aiello *et al.,* 1995; Aiello *et al.,* 1998). In the light of these findings, CORM-A1 can activate K_V channels through activation of the cGMP/ PKG pathway which might phosphorylate the channel protein directly, or indirectly by cross activation of the cAMP/PKA pathway (Aiello *et al.*, 1995; Aiello *et al.*, 1998). Alternatively, CORM-A1, independent of cGMP, might directly interact with K_V channels through mechanisms yet to be defined.

In conclusion, knowing that there is no drug which acts with exclusive specificity, and in the light of published studies, we believe we have justified and confirmed the importance of compounds used in this study to assess the involvement of sGC/cGMP pathway and K^+ channels in mediating the vasorelaxing properties of

CORM-3 and CORM-A1. The current results have shown that sGC is a cellular target for both CORM-3 and CORM-A1, whereas each CO-RM activates different K^+ channel subtypes. CORM-3 and CORM-A1 activated K_{ATP}, and K_V channels respectively. Each CO-RM might modulate each subunit directly or indirectly through sGC/cGMP pathway activation.

7.3 Comparison between the vascular activity elicited by water soluble CO-RMs and the CO delivered by other methods

The attitude toward CO has changed dramatically over the last two decades from nothing but silent killer to a gas with diverse biological activity and therapeutic potential (Johnson *et al.*, 1999; Morse and Choi, 2002). HO enzyme which catalyzes the endogenous formation of CO from heme molecules has been expressed in almost all life forms, ranging from prokaryotic bacteria and fungi to plants and human (Wagener *et al.*, 2003). Actually, there are no enzymes identified to date that are affected by so numerous stimuli of diverse nature as is HO-1, the inducible form of HO (Maines *et al.,* 1997). The CO, endogenously derived or exogenously applied, is involved in cellular signalling in cardiovascular, gastrointestinal, liver, respiratory, inflammatory and nervous system (Wang *et al.*, 1997a; Zakhary *et al.*, 1997; Goda *et al.*, 1998; Otterbein *et al.,* 2002; Gibbons and Farrugia, 2004). In both systemic and pulmonary circulation CO plays an important role in regulating vascular tone under physiological and pathological conditions (Wang *et al.,* 1998; Villamor *et al.*, 2000; Zhao *et al.*, 2002). It attenuates vasoconstriction responses and induces vasodilatation of diverse blood vessels with different diameters and from different species through different mechanisms. Among different mechanisms proposed to elucidate the vasorelaxing properties of CO, activation of sGC/cGMP pathway and BK_{Ca} channels have received significant interest (Wang *et al.,* 1998; Zhang *et al.,* 2001b; Zhao *et al.,* 2002; Morse and Choi, 2005). However, despite the progress made in understanding the pathophysiological and therapeutic potential of CO over the last two decades, it remains incomparable to that of NO gas which has been extensively investigated and found its way into clinical practice to treat diverse pathological conditions (Moncada and Higgs 1995; Cohen *et al.,* 1999; Motterlini *et al.,* 2002b). The experimental studies on NO have been largely facilitated by the development of a wide variety of organic compounds that spontaneously release NO and can be easily acquired to produce physiological or pathophysiological functions of NO (Feelisch *et al.,* 1998; Ignarro *et al.,* 2002; Motterlini *et al.,* 2003). Undoubtedly, discovery of similar compounds that can carry and deliver CO into vascular tissue in safe, measured and controllable way will extend our understanding of the chemistry of CO, its interaction with intracellular targets, and its importance in physiology and medicine (Motterlini *et al.,* 2002b; Motterlini *et al.,* 2003). Recently, Dr Motterlini has discovered the ability of some transitional metal carbonyl compounds to release CO into physiological solutions. These compounds have been termed CO-releasing molecules (CO-RMs) (Motterlini *et al.,* 2001; Motterlini *et al.,* 2002a). Dimanganese decarbonyl (CORM-1) and tricarbonyldichloro ruthenium (II) dimer (CORM-2), proved to be pharmacologically active with functional effects that are similar to CO gas, particularly vasorelaxation and suppression of acute hypertension. However both molecules are soluble only in an organic solvent such as DMSO, and CORM-1 requires light to induce CO release which raises justified doubts about their application *in vivo*. Water solubility is of great importance to drugs and there is a universal desire in drug discovery to increase water solubility or to decrease hydrophobicity (Berger *et al.,* 2004). Generally the more water-soluble the drugs are, the better their bioavailability when they are administrated orally (McFarland *et al.,* 2003; Berger *et al.,* 2004). Therefore, it is an advantage in designing new drugs to be able to estimate their solubility before their preparation as potential new compounds might be removed from consideration if their predicted solubility makes them hopeless candidates (McFarland *et al.,* 2003; Berger *et al.,* 2004). For these reasons our group intensified their work to discover new water soluble CO-RMs that overcome the solubility problem of the first generation of CO-RMs. As a result, CORM-3, CORM-A1, and CORM-319, three structurally different water soluble CO releasing molecules have been identified and produced (Foresti *et al.,* 2004; Motterlini *et al.,* 2005). Tricarbonylchloro(glycinato) ruthenium(II) (CORM-3) is a ruthenium based metal carbonyl complex that has been shown to spontaneously and relatively rapidly release CO into biological buffers ($t_{1/2}$ ~1-5 min) (Foresti *et al.*, 2004). Sodium boranocarbonate $Na_2 [H_3 BCO_2]$ (CORM-A1) is another water soluble molecule that releases CO spontaneously into physiological solutions (Motterlini *et al.*, 2005). However, the rate of CO release from CORM-A1 is slower than that from CORM-3 ($t_{1/2}$~21 min) (Motterlini *et al.*, 2005). The last molecule in this group is iron based ($[Fe(CO)_4(\eta^3\text{-}C_3H_4Me)]BF_4$) (CORM-319) which quickly releases CO into physiological solutions ($t_{1/2}$ ~ 1 min) when assessed by amperometric CO sensor (unpublished data from our group).

In the study presented here we tested the hypothesis that water soluble CORM-3, CORM-A1, and CORM-319, like CO, can elicit vasorelaxation in precontracted aortic rings. Additionally, we also tried to confirm the hypothesis that CORM-3 and CORM-A1 mediate their vasorelaxation through activation of sGC/cGMP and or BK_{Ca} channels. The results of this project substantiate the first part of this hypothesis as all CO-RMs tested in this study induced concentration dependant vasorelaxation. However, the mechanisms by which CORM-3 and CORM-A1 function do not fit completely with our hypothesis. Both of them activated the sGC/cGMP pathway but K_V and K_{ATP} channels, not BK_{Ca}, were the cellular targets of CORM-A1 and CORM-3 respectively.

The current results indicate that CO liberated from these molecules is responsible for their vasorelaxing effect as the inactive forms (iCO-RMs), which do not release CO, did not have any significant effect on vascular tone. Additionally, CORM-3 and CORM-319 which have a fast pattern of CO release induced rapid vasorelaxation which reached a maximum within 10 min, whereas CORM-A1 which has a slower pattern of CO release elicited gradual relaxation which reached maximum after around 30 min. These findings show that the vasorelaxing pattern of the tested CO-RMs reflects their kinetics of CO release.

In this study we showed that similar to HO-derived or externally applied CO gas, both CORM-3 and CORM-A1 activate sGC which in turn catalyzed the production of cGMP, an important intracellular second messenger that mediates the relaxation of vascular smooth muscle cells. On the other hand, the absence of a significant role for BK_{Ca} channels in our project, does not agree with other studies elsewhere which reported that CO, either endogenously produced or externally applied, primarily targets BK_{Ca} channels in vascular tissue (Wang *et al.*, 1997a; Wang *et al.*, 1997b; Naik and Walker, 2003; Wang and Wu, 2003; Dubuis *et al.*, 2005 ; Jaggar *et al.*, 2005). There are three possible explanations for this discrepancy between our results and these studies. Firstly, although the findings of other studies confirm the role of BK_{Ca} channels, they did not rule out the role of vascular K_V, K_{ATP} channels as target for CO activity. Secondly, it is well known that different K^+ channel subtype expression and dominance is highly tissue specific (Nelson and Quayle, 1995; Coghlan *et al.* 2001; Cao *et al.,* 2002; Jackson *et al.*, 2005). Furthermore, the expression and function of various K^+ channel subtypes in the same cultured vascular cells might vary in response to *in vitro* culture conditions (Tang and Wang, 2001). For example BK_{Ca} current is predominant whereas K_V current is a minor component of total outward K^+ current in freshly dissociated cultured SMCs of rat tail artery. In contrast the K_V current was found to the predominant in the same cells after 24 hours incubation (Tang and Wang, 2001). Therefore, the role played by BK_{Ca} channels in mediating the vasorelaxing properties of CO in rat tail artery, mesenteric artery and pulmonary arteries might reflect the predominance of this K^+ channels subtype in these vascular bed. Regarding aortic arteries, at least in male Wistar rats. K_V channels not charybdotoxin-sensitive channels were shown to play an important role in the regulation of excitability and contractility of rat aorta (Tammaro *et al.*, 2004). Therefore, the activation of K_V and K_{ATP} channels by CORM-A1 and CORM-3 respectively in isolated rings of adult male Sprague Dawley rats might be a reflection of the expression and function of these channels in aortic SMCs. Generally speaking, K^+ channels are the most diverse and sophisticated ion channels in mammalian cells and their role in mediating the effect of vasorelaxing or vasoconstricting agents should be analysed carefully (Cao *et al.,* 2002; Mannhold et al., 2004; Tammaro *et al.*, 2004).

Lastly, the pattern of CO delivery to vascular tissue might explain the variation regarding the role of K^+ channel subtypes between our results and those published by other groups. Additionally it might explain the difference between the mechanisms of action between CORM-3 and CORM-A1. Here, we tested the vascular action of CO gas delivered by water soluble CO-releasing molecules while the others assessed the effect of HO-derived or externally applied CO gas. It is noteworthy that different effects and mechanisms of action were reported between endogenously formed and externally applied CO gas (Naik and Walker, 2003; Hristov *et al.*, 2004). For example endogenous CO derived from HHL, the HO substrate, elicited sGC independent vasorelaxation in mesenteric arterioles, whereas exogenous CO-induced vasorelaxation was sGC dependant (Naik and Walker 2003). Interestingly, iberiotoxin, the BK_{Ca} blocker, attenuated the vasorelaxing effect of both endogenous and exogenous CO. In another study conducted on isolated smooth muscle cells of portal vein, both hemin, the HO substrate, and external CO had similar effects, both increased K_{Ca} current, although hemin was significantly more potent than CO (Hristov *et al.*, 2004). Inexplicably, the effect of external CO was sGC dependant, whereas hemin effect was sGC independent. Furthermore iron administration or chelating activate or suppresses K_{Ca} channels respectively. Therefore, it was concluded that the stimulatory effect of hemin on K_{Ca} was not due to CO production (Hristov *et al.*, 2004). These two studies confirm that the methods of CO delivery into vascular tissue could affect its pharmacological action. Hence, the pattern of CO release by CORM-3 and CORM-A1 (fast versus slow) might explain the difference in their cellular target. For unknown reasons which need further clarification, it seems that CO released rapidly from CORM-3 activates K_{ATP} channel subtypes whereas CO liberated slowly from CORM-A1 preferred K_V channels. Indeed we do not know why the fast pattern of CO release activates K_{ATP} channels, whereas slow pattern stimulates the other K^+ channel subgroup. Physical, electrochemical, or unknown mechanisms might lie behind this effect. Surely, a lot of work is needed to understand this effect; however we think that this sophisticated work is beyond the scope of this thesis.

Another important finding of our study is that CORM-3 and CORM-A1 at concentrations higher than those which induced maximum vasorelaxation have no toxic effect on cultured aortic SMCs. This result could help to design effective carbon monoxide releasing molecules that can be used safely *in vivo* and clinical practice. Such molecules might be of great value to treat diverse vascular diseases such as hypertension, coronary heart disease, cerebral and peripheral vascular disease. Although CORM-319 elicited mild damage at 50 μM which induced moderate vasorelaxation *ex vivo*, it induced remarkable cytotoxicity at 100 μM and completely destroyed the cultured aortic SMCs at 500 μM. However, CORM-319 is notably safe if compared with other iron containing CO-RMs tested in this project. These water insoluble molecules which have very similar chemical structure to CORM-319, completely damaged aortic SMCs at concentrations as low as 10 μM. The water insolubility and extreme toxicity of these CO-RMs show how a minor difference in the chemical structure of iron containing CO-RMs has great impact on their pharmacological activity.

Another important finding in the study presented here is that the toxicity level of a given concentration of CO is highly dependant on the chemical structure of the releasing molecules. For example, when 500 μM CORM-319 was applied to cultured SMCs, it induced around 100 % loss of cell viability, while only 40% damage was imposed by iCORM-319, the inactive form which does not release CO. This means that the 500 μM of CO gas released by CORM-319 is likely responsible for this immense toxicity. However, 500 μM of CORM-A1 which release the same amount of CO, had insignificant effect on the cell viability. These findings highlight the great importance of developing various CO releasing molecules with various structures (ruthenium, boron, or iron based) in understanding the biochemical aspects of CO.

In conclusion, our study leads us to substantiate the hypothesis that the water soluble CORM-3, CORM-A1 and CORM-319 can induce vasorelaxation of precontracted aortic rings, and this effect is most likely mediated through their released CO gas. Both CORM-3 and CORM-A1 stimulate the sGC/cGMP pathway. However, unlike HO derived or exogenously applied CO gas, CORM-3 activates K_{ATP} channel subtypes whereas CORM-A1 activates K_V subtypes. Additionally, the data presented here demonstrate the great impact imposed by the kinetic of CO release and change in chemical structure on the pharmacological activity of CO-RMs. Finally, these results also show that the CORM-3 and CORM-A1 are relatively safe when applied to cultured aortic SMCs at concentrations higher than those which induced significant vasorelaxation. If this safety is supported by further *in vitro* and *in vivo* toxicological screening, CORM-3 and CORM-A1 might be of great therapeutic value as vasorelaxing drugs. On the other hand, the noticeable toxicity of CORM-319 at 100 μM might raise questions about its future as an optimal medicine. A modification on the chemical structure of CORM-319 might resolve any potential cytotoxicity.

7.4 Conclusions, Reflections and Future Perspectives

CO is an important regulator of vascular tone under both physiological and pathological conditions. Therefore, the pharmaceutical modulation of vascular CO levels has been speculated to be of great importance in treating vascular disease (Morse and Choi, 2005; Abraham and Kappas, 2005). In this project we confirmed that the water soluble CORM-3, and CORM-A1 molecules meet the criteria of pharmacological active CO carriers that can spontaneously and safely release CO into physiological solutions to modulate vascular tone. Undoubtedly, the water solubility of these CO-RMs is of great pharmaceutical advantage over the first generation of CO-RMs which were soluble only in organic solvents. Our attempt to diversify the collection of CO-RMs that have a variety of chemical structure (ruthenium, boron, or iron based) and patterns of CO release (fast vs. slow release) will help to elucidate the biochemical characteristics of CO, and the cellular targets that are responsive to CO and will facilitate the therapeutic delivery of CO.

The current work is a significant step in a long journey started when endogenous production of CO was discovered for the first time and will be completed by optimal therapeutic application of CO in vascular and other diseases, such as hypertension and coronary artery disease. The involvement of K^+ channels and other ion channels or cellular pathways need to be further examined in other vascular beds and through additional techniques, for example patch-clamp technique. The efficacy, tissue distribution and elimination of the CO-RMs presented in this study must be examined in different animal species *in vivo*. Last but not least, comprehensive toxicological studies are essential to approach optimal therapeutic concentrations of this gas with a bad history.

Whether the molecules presented in this study in their current shape reach the hospital bedside or not, we deeply believe that they are a breakthrough in the biomedical field of CO and will provide the base for many other studies investigating the biology of CO.

LIST OF PUBLICATIONS

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9 APPENDIX

The pattern of CO released from CORM-319, CORM-3 and CORM-A1 was assessed using an amperometric CO sensor (**Figure 9.1, 9.4**) and myoglobin assay (**Figure 9.2, 9.3 and 9.5**). As mentioned in the text the chemical structure of a tested CO-RM should be considered in interpreting the results of any applied assay. For example, CORM-319 has a fast pattern of CO release when assessed by CO sensor (**Figure 9.1**), whereas it has a slower pattern of CO release when assessed by the myoglobin assay (**Figure 9.2**). In contrast, CORM-3 hardly release any CO when assessed by CO sensor (data not shown), while it had a fast pattern of CO liberation when tested by myoglobin assay (**Figure 9.3**). CORM-A1 which is a very pure salt, has similar kinetics of CO release whether measured by CO sensor (**Figure 9.4**) or myoglobin assay (**Figure 9.5**).

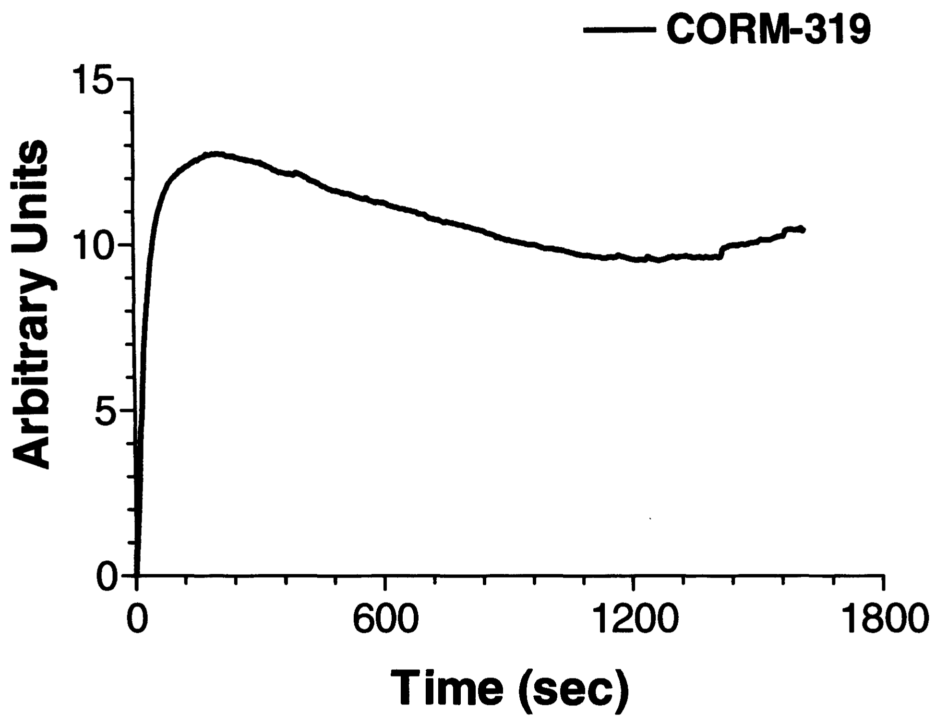

Figure 9.1 Quantification of CO liberated from CORM-319 using CO electrode (from Sandip Bains).

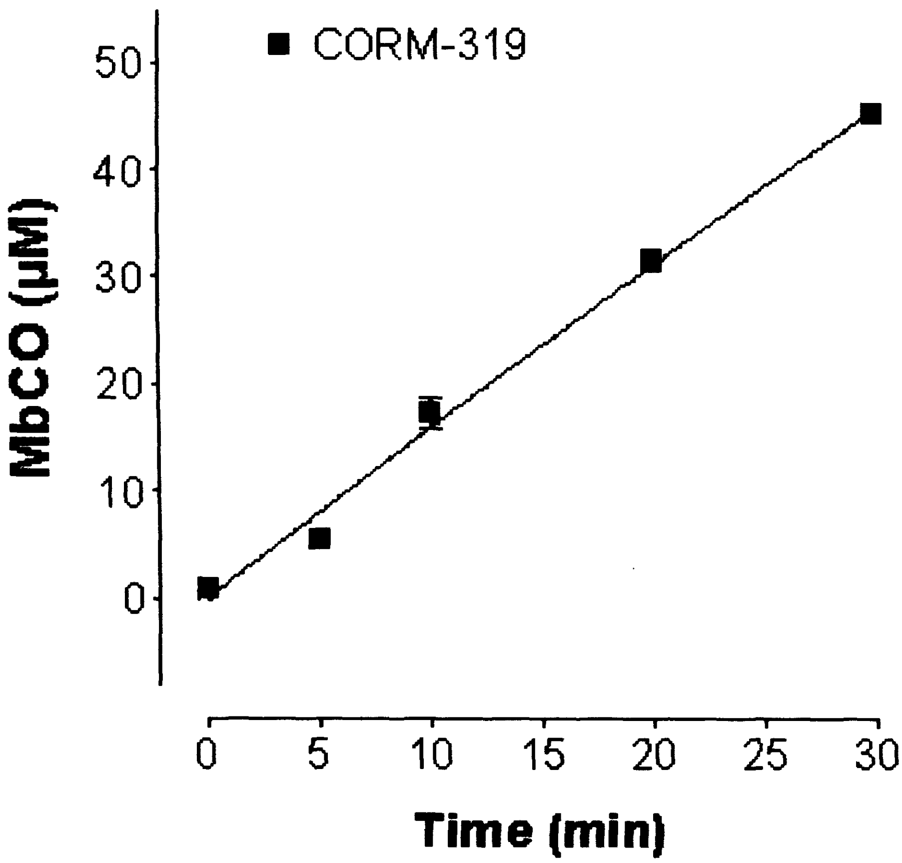

Figure 9.2 Quantification of CO liberated from CORM-319 using the myoglobin assay (from Dr Philip Swale).

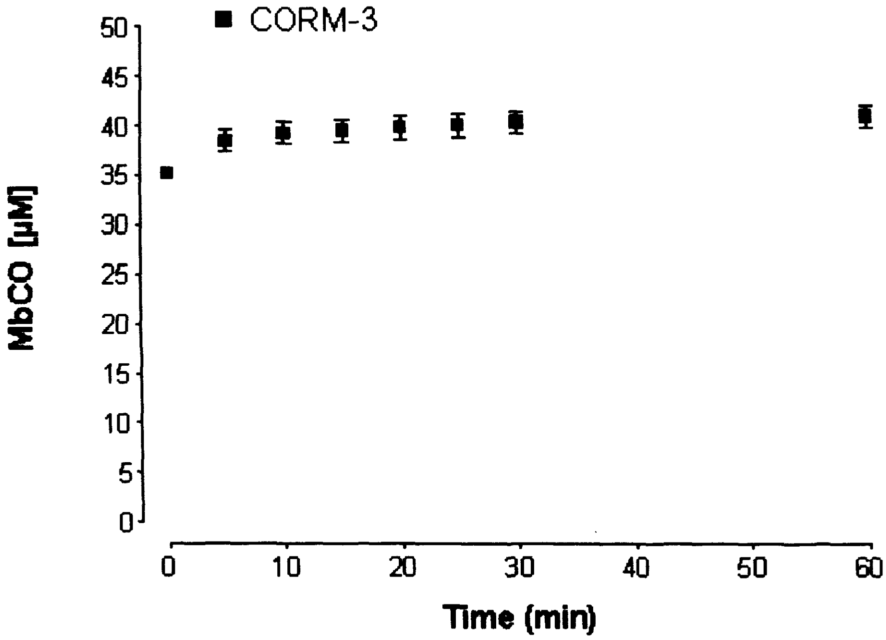

Figure 9.3 Quantification of CO liberated from CORM-3 using myoglobin assay (from Dr Philip Swale).

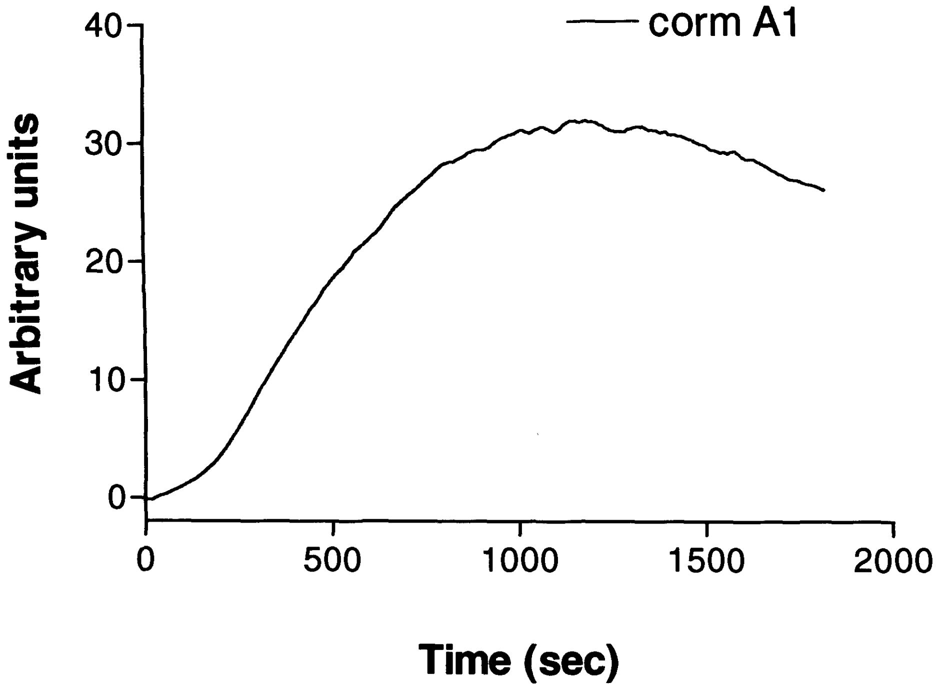

Figure 9.4 Quantification of CO liberated from CORM-A1 using an amperometric CO sensor (from Sandip Bains).

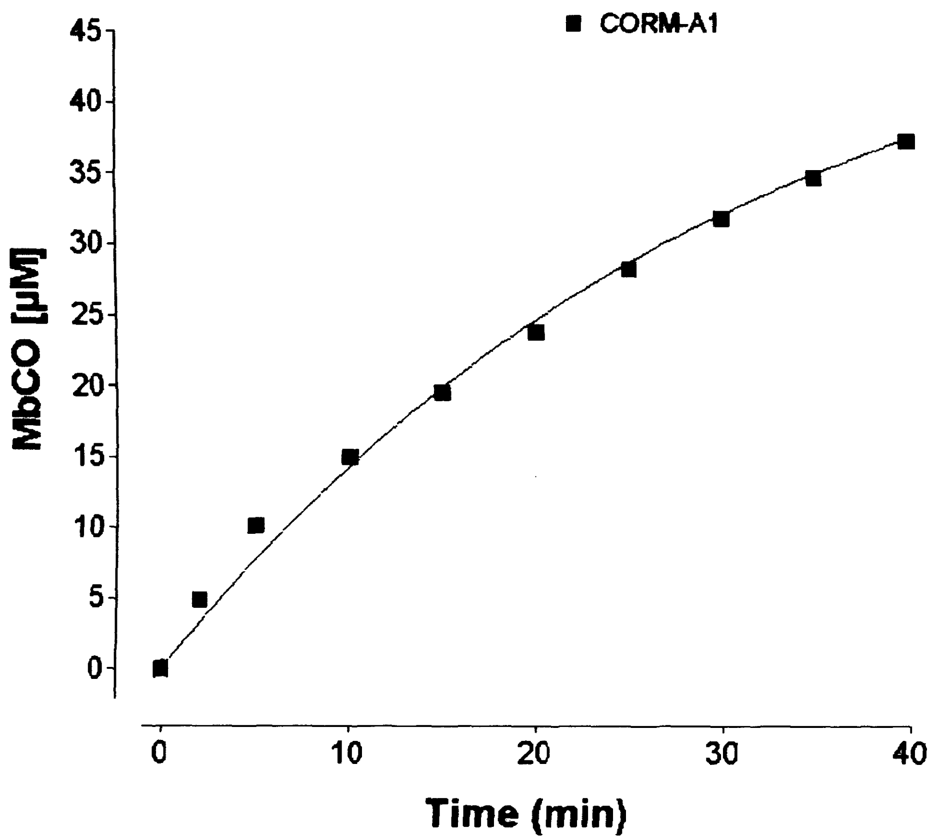

Figure 9.5 Quantification of CO liberated from CORM-A1 using a myoglobin assay (from Dr Philip Sawle).